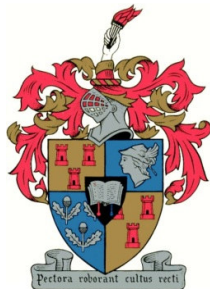


**CRITICAL EVALUATION OF THE ACCURACY OF THE ENUMERATION METHODOLOGY OF  
COLIFORMS AND *E. COLI* IN WATER FROM RIVERS USED FOR THE IRRIGATION OF  
FRESH PRODUCE**

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in the Department of Food Science  
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at  
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**December 2012**

### **Declaration**

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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## Abstract

The accuracy of methods for the enumeration of coliforms and *Escherichia coli* present in river water intended for the irrigation of fresh produce has been critically evaluated to determine whether the results of the traditional method were reliable in indicating faecal pollution. The potential of rapid alternative methods were also explored.

Baseline monitoring of the Berg River showed the presence of potential pathogens such as *Salmonella*, and also that *E. coli* levels exceeded international guidelines for the safe irrigation of minimally processed foods (MPFs) in 20.5% of cases, which indicated faecal intrusion. An exploratory study into the use of microbiological and physico-chemical parameters in predicting *E. coli* numbers, as a rapid alternative to direct enumeration, was conducted. These measurements, neither individually nor in combination, could accurately predict the *E. coli* numbers.

The rapid method Colilert-18 was compared against multiple tube fermentation (MTF) for the enumeration of coliforms and *E. coli*. Spearman rank correlation coefficients showed that Colilert-18 had acceptable ( $r^2=0.69$ ) and fair ( $r^2=0.74$ ) correlations with MTF for coliform and *E. coli* enumeration, respectively. Bland and Altman statistics were used to determine pollution influence, and Colilert-18 showed increasing disagreement with MTF at very high concentrations of coliforms and *E. coli*.

Bacterial isolates obtained from MTF reactions were identified using biochemical and mass spectrometry methods. These identifications revealed that the greatest contributors to inaccurate coliform enumeration by MTF were false negative coliforms which fail to produce gas from lactose. Numerical biochemical data suggested that these isolates may be able to use other carbohydrates preferentially over lactose. Inaccurate *E. coli* enumeration was caused by *E. coli* strains which could not utilise lactose or 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG), as well as non-*E. coli* isolates which were able to hydrolyse MUG. The method of transfer of bacteria between MTF media was also identified as problematic for accuracy.

Monoplex polymerase chain reaction (PCR) differentiation of MTF isolates showed that detection of the *uidA* gene showed the greatest accuracy in the detection of *E. coli*, while the multiplex PCR protocol for detecting diarrheagenic *E. coli* pathotypes identified one strain of enteroaggregative *E. coli* (EAEC).

A qualitative methodological risk classification was used, in combination with the individual reactions of MTF isolates, to elucidate their contribution to enumeration inaccuracy and to evaluate the effect of MUG and Levine-eosin methylene blue (L-EMB) agar. The classification indicated that inaccurate enumeration of *E. coli* was more problematic than that of coliforms, but revealed that the exclusion of MUG from MTF may increase the accuracy of *E. coli* enumeration. The omission of L-EMB would have very little effect on *E. coli* enumeration accuracy.

This work confirmed that MTF is fairly reliable in the enumeration of coliforms and *E. coli*. Inaccuracies are primarily attributable to atypical organisms which are considered to make up a small proportion of the total bacterial population. Colilert-18 was shown to be an acceptably accurate alternative, and its rapid production of results can be highly advantageous in the monitoring of irrigation water used for MPFs.



## Opsomming

Die akkuraatheid van metodes vir die telling van kolivorme en *Escherichia coli* in rivier water, gebruik vir besproeiing van vars produkte, is krities geëvalueer om vas te stel of die resultate van tradisionele metodes betroubaar was in die aanduiding van fekale besoedeling. Die potensiaal van snelle alternatiewe metodes is ook ondersoek.

Basislyn monitering van die Berg River het aangedui dat potensiële patogene soos *Salmonella* teenwoordig is, en dat *E. coli* vlakke internasionale riglyne vir die veilige besproeiing van minimaal geprosesseerde voedsels (MGVs) oorskry het in 20.5% van gevalle, wat dui op fekale besmetting. 'n Ondersoekende studie van die bruikbaarheid van mikrobiologiese en fisies-chemiese parameters in die voorspelling van *E. coli* getalle, as snelle alternatief tot direkte telling, is uitgevoer. Hierdie metings kon nie, individueel of in kombinasie, akkurate voorspellings van *E. coli* getalle maak nie.

Die snelle metode Colilert-18 is vergelyk met veelvoudige buis fermentasie (VBF) in die telling van kolivorme en *E. coli*. Spearman rang korrelasie koëffisiënte het aangetoon dat Colilert-18 aanvaarbare ( $r^2=0.69$ ) en goeie ( $r^2=0.74$ ) korrelasies met VBF gehad het vir kolivorm en *E. coli* tellings, respektiewelik. Bland en Altman statistiek is gebruik om die invloed van besoedeling te bepaal, en Colilert-18 het afnemende ooreenstemming met VBF getoon by baie hoë kolivorm en *E. coli* konsentrasies.

Bakteriële isolate verkry vanaf VBF reaksies is geïdentifiseer met behulp van biochemiese en massa spektrometrie metodes. Hierdie identifikasies het getoon dat vals-negatiewe kolivorme, wat nie gas vanaf laktose kan produseer nie, die grootste bydraende faktor is in onakkurate kolivorm telling deur VBF. Biochemiese data het voorgestel dat hierdie isolate moontlik ander koolhidrate by voorkeur bo laktose gebruik. Onakkurate *E. coli* tellings is veroorsaak deur *E. coli* isolate wat nie laktose of 4-metielumbelliferiel- $\beta$ -D-glukuronied (MUG) kon verbruik nie, sowel as nie-*E. coli* isolate wat wel MUG kon hidroliseer. Die oordrag-metode van bakterieë tussen VBF media is ook geïdentifiseer as problematies.

Monopleks polimerase ketting reaksie (PKR) onderskeiding van VBF isolate het aangedui dat opsporing van die *uidA* geen die grootste akkuraatheid vir die opsporing van *E. coli* het, terwyl die multipleks PKR protokol vir die opsporing van diarree-veroorsakende *E. coli* patotipes een stam van entero-aggregerende *E. coli* (EAEC) geïdentifiseer het.

'n Kwalitatiewe metodologiese risiko klassifikasie is gebruik, in kombinasie met die individuele reaksies van VBF isolate, om hul bydrae tot telling onakkuraatheid vas te stel. Die effek van MUG en Levine-eosien metileenblou (L-EMB) agar is ook geëvalueer. Daar is bevind dat die onakkurate telling van *E. coli* meer problematies is as dié van kolivorme, maar ook dat die uitlating van MUG by VBF die akkuraatheid van *E. coli* tellings kan verhoog. Die uitlating van L-EMB agar sal 'n ignoreerbare effek hê op *E. coli* telling akkuraatheid.

Hierdie werk het bevestig dat VBF akkuraat is in die telling van kolivorme en *E. coli*. Onakkuraathede word primêr toegeskryf aan atipiese organismes, wat beskou word as 'n klein proporsie van die totale bakteriële bevolking. Colilert-18 is 'n aanvaarbaar akkurate alternatief, en die metode se produksie van snelle resultate kan hoogs voordelig wees in die monitering van MGVs besproeiingswater.

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## List of Abbreviations

ACC	Aerobic colony count
AFNOR	Association Française de Normalisation (French Standardization Association)
API	Analytical Profile Index
ATCC	American Type Culture Collection
BGLB	Brilliant green lactose bile
BPW	Buffered peptone water
BTS	Bacterial test standard
CDC	Centers for Disease Control
COD	Chemical oxygen demand
DAEC	Diffusely adherent <i>E. coli</i>
dNTP	Deoxyribonucleotide triphosphate
DoA	Department of Agriculture, now DAFF
DWA	Department of Water Affairs after 2008
DWAF	Department of Water Affairs and Forestry before 2008
EAEC	Enterotoxigenic <i>E. coli</i>
EAHEC	Enterotoxigenic-haemorrhagic <i>E. coli</i>
EC (broth)	<i>Escherichia coli</i> broth
EHEC	Enterohaemorrhagic <i>E. coli</i>
EIEC	Enteroinvasive <i>E. coli</i>
EPEC	Enteropathogenic <i>E. coli</i>
EPS	Exopolysaccharides
ETEC	Enterotoxigenic <i>E. coli</i>
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FIB	Faecal indicator bacteria
HCCA	Alpha ( $\alpha$ )-cyano-4-hydroxycinnamic acid
HIV	Human immunodeficiency virus
HUS	Haemolytic uremic syndrome
ICC	Intra-class correlation
ISO	International Organization for Standardization
L-EMB (agar)	Levine-eosin methylene blue agar
LST	Lauryl sulphate tryptose
LTB	Lauryl tryptose broth
MALDI-TOF MS	Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

mFC	Membrane faecal coliform
MLGA	Membrane lactose glucuronide agar
MLSB	Membrane lauryl sulphate broth
MPF(s)	Minimally processed food(s)
MPN	Most probable number
MSP	Main spectral projection
MUF	Methylumbelliferone
MUG	4-methylumbelliferyl- $\beta$ -D-glucuronide
NA	Nutrient agar
NB	Nutrient broth
NMG	Not metallic green
NMMP	National microbial monitoring programme
ONPG	ortho ( <i>o</i> )-nitrophenyl- $\beta$ -D-galactopyranoside
PCA	Plate count agar
PCR	Polymerase chain reaction
PSS	Physiological saline solution
RV	Rappaport-Vassiliadis
SABS	South African Bureau of Standards
SANS	South African National Standards
SC	Selenite cysteine
SCM	Standard culture mix
SEM	Standard Error of Measurement
TBE	Tris-borate-EDTA
TTC	Triphenyltetrazolium chloride
TSA	Tryptose soy agar
USEPA	United States Environmental Protection Agency
VBNC	Viable but non-culturable
WHO	World Health Organization
WRC	Water Research Commission
XLD	Xylose lysine desoxycholate

## CHAPTER 1

### INTRODUCTION

South Africa is a semi-arid country which does not possess water in the same abundance as many other natural resources. The average rainfall is 500 mm per annum (WRC, 2008) in comparison with the 860 mm annual world average (Friedrich *et al.*, 2009). This low annual average is the major reason why most produce in South Africa is irrigated, demanding 52% of the country's water resources (Jackson *et al.*, 2009). Of these water resources, river water is most commonly used for irrigation since it is the cheapest and most readily available water source. Unfortunately, the utilisation of river water is not limited to agricultural and industrial processes. These resources are also inadvertently used as sewage canals when faecal waste from settlements with inadequate sanitation are washed or, more directly, deposited into rivers and the surrounding environment (Jackson *et al.*, 2007). Agricultural activities involving the production of livestock further contaminate the water resources with faecal pollution and enteric pathogens from animal waste.

Apart from the health implications of polluted water resources, which contribute to 5.7% of the global burden of disease (Shannon *et al.*, 2007), the irrigation of produce which will be consumed raw or after minimal processing poses a risk for consumer health. This is due to the transfer of pathogens from the contaminated irrigation water onto the fresh produce, as reported by Lu *et al.* (2004). Contaminated produce have been implicated in many outbreaks; including uncooked spinach (King, 2006; Abadias *et al.*, 2008), lettuce (Velázquez, 2009) and sprouts (Johnston *et al.*, 2005) contaminated with *E. coli* O157:H7, ready-to-eat salads (Velázquez, 2009) contaminated with *E. coli* O157:H7 and *Staphylococcus aureus*, and most recently fenugreek seeds carrying enteroaggregative-haemorrhagic *E. coli* (EAHEC) (Brzuszkiewicz *et al.*, 2011). These health implications are one of the important driving forces for determining the level of faecal contamination in water before it is used for irrigation. It is also necessary to analyse the irrigated products to ensure that no threat is posed to the health of the consumer by faecally-associated pathogens present on the surface of minimally processed foods (MPFs).

For the determination of the level of faecal contamination in a sample, *Escherichia coli* is considered to be the best indicator organism for water microbiology (Edberg *et al.*, 2000). Consequently, the methods for enumerating this organism have received the attention of many researchers attempting to find the method which yields the most accurate results. Two of the traditional methods for the detection and enumeration of coliforms and *E. coli* are the membrane filtration and multiple tube fermentation (MTF) methods (Rompré *et al.*, 2002). These have been approved by the United States Environmental Protection Agency (USEPA) and the French Standardization Association (AFNOR) (Rompré *et al.*, 2002). The MTF method; despite its associated high costs, laborious protocol and four-day waiting period before results are available (Maheux *et al.*, 2008); has remained the preferred method for accurate results. This is attributable

to its semi-statistical quantification of the number of test organisms in the sample with the help of most probable number (MPN) tables (Edberg & Edberg, 1988; Christensen *et al.*, 2002), and also the numerous selective hurdles which are incorporated within the media used in the method. Therefore, this method is considered to be the most accurate enumeration method for *E. coli* and is subsequently considered to give the closest approximation of health risk that can be obtained through testing for this organism.

Despite the perceived accuracy of the MTF method, many problematic observations have been reported. The inability to produce gas from lactose in the media (designated “anaerogenic” throughout this dissertation) by both coliforms (LeChevallier *et al.*, 1983) and *E. coli* (Meadows *et al.*, 1980; Evans *et al.*, 1981b; Fricker *et al.*, 1997; Leclerc *et al.*, 2001) can cause under-estimation of coliforms and *E. coli*. Synergy between non-target organisms, such as *Proteus* and enterococci (Schiff *et al.*, 1970), can cause reactions which lead to false positives and, consequently, over-estimation. The ability of organisms other than *E. coli* to hydrolyse the compound 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG), and the inability of some strains of *E. coli* to do so, can lead to further incorrect enumeration with this method. In addition, the presence of autochthonous heterotrophic bacteria at concentrations exceeding 500 cfu.mL<sup>-1</sup> (Evans *et al.*, 1981a) and interference by Gram negative bacteria which do not belong to the coliform grouping (Edberg & Edberg, 1988) can also impact negatively on the accuracy of the method. To amend this inaccuracy, further validation has been recommended to reduce the impact of false positives (Edberg & Edberg, 1988). This is done as part of the complete MTF protocol through the streaking of positive *E. coli* (EC) broth tubes on Levine-eosin methylene blue (L-EMB) agar (Christensen *et al.*, 2002). However, the occurrence of both false positives and false negatives remains disquieting for the accuracy of the method and, furthermore, the dualistic effects of MUG and L-EMB agar on the accuracy of the method is unknown.

Apart from the traditional methods for the detection and enumeration of coliforms and *E. coli*, advances in science and technology have seen the emergence of a variety of new methods for analysing coliforms and *E. coli*. One alternative method for the detection and enumeration of coliforms and *E. coli* is the Colilert-18 method. This method detects the presence of the  $\beta$ -D-galactosidase enzyme in coliforms and *E. coli*, and the presence of the  $\beta$ -D-glucuronidase enzyme in *E. coli*. The detection is reliant on the breakdown of *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) and MUG by  $\beta$ -D-galactosidase and  $\beta$ -D-glucuronidase, respectively. Comparative studies between this method and other methods have shown higher recovery rates for *E. coli* numbers against membrane faecal coliform (mFC) agar (Wohlsen *et al.*, 2008) and membrane lactose glucuronide agar (MLGA) (Fricker *et al.*, 2010), and similar and higher sensitivity for *E. coli* and coliforms respectively when compared with MTF (Kämpfer *et al.*, 2008; Eckner, 1998) in drinking water.

Molecular methods such as polymerase chain reaction (PCR) have become more accessible for routine analyses due to a considerable reduction in operating costs. In addition, the



method does not require cultivation before detection of the target organism (Ashbolt *et al.*, 2001), and it is sensitive, specific and rapid (Girones *et al.*, 2010). The detection of *E. coli* through a PCR assay detecting the *uidA* gene has been recommended as a more reliable method of *E. coli* detection than testing for the presence of the  $\beta$ -D-glucuronidase enzyme (Bej *et al.*, 1991; Heijnen & Medema, 2006). This molecular method can also provide additional information regarding the *E. coli* strains which are detected in the sample through a pathotype PCR. This assay can distinguish the diarrheagenic pathotypes of *E. coli* from the commensal strains, and thereby give a better indication of the risk posed by the faecal contamination since these pathotypes can lead to serious or fatal (Fagan *et al.*, 1999) enteric diseases.

It is of great importance that the quality of South Africa's water resources is kept high, to avoid outbreaks related both to direct contact with water and through the consumption of irrigated MPFs. In order to improve or maintain the quality of the water, the current state of South Africa's water resources should first be determined. In addition, the methods which are used to determine the extent of faecal pollution in the water should be evaluated to find the most accurate method for enumerating coliforms and *E. coli* from river water.

The main objective of this study is to critically evaluate the accuracy of the methodology used to enumerate coliforms and *E. coli* in water from rivers which are used for the irrigation of fresh produce. This objective will be reached by firstly determining the baseline microbiological and physico-chemical quality of water from the upper Berg River, as an example of a South African river which is used for the irrigation of fresh produce. These measurements will then be used to conduct an assessment into the feasibility of using alternative microbiological, physical and chemical parameters as predictors of *E. coli* numbers in water from this river. This assessment is done since these tests could potentially provide predictive results in far less time than is required to directly enumerate *E. coli*, which could lead to quicker corrective and preventative action. A direct method for the enumeration of coliforms and *E. coli*, Colilert-18, will also be evaluated as a rapid alternative to MTF in the analysis of South African river water, using water from four South African rivers with varying levels of faecal pollution. The phenotypic characterisation and identification, using biochemical attributes and ribosomal proteins, will be performed on isolates obtained from various steps of MTF analyses done on river water. These results can provide insights into which organisms can cause under- or over-estimation with this method, and also how these inaccuracies manifest as observable reactions in MTF. The MTF isolates will also be used to evaluate PCR protocols for distinguishing *E. coli* from coliforms, and determine whether they corroborate phenotypic identification of the isolates. The impact of these findings on MTF accuracy will be discussed. A PCR protocol for differentiating between diarrheagenic *E. coli* pathotypes will also be evaluated. The numerical data obtained from biochemical and ribosomal protein characteristics of the MTF isolates will be used to construct dendrograms based on similarity and dissimilarity, thereby determining whether numerical data can differentiate between organisms which cause divergent reactions in the MTF method. The individual contribution of each isolate to the MTF will

be determined through the inoculation of each organism individually. These reactions will then be used in a qualitative methodological risk classification system which will categorise the risk for inaccuracy posed by each isolate. The benefits and detriments of including MUG and L-EMB agar in this method will also be evaluated using this methodological risk classification. Finally, the experimentally obtained information arising from the aforementioned work will be used to make recommendations to both the scientific and industrial communities. These recommendations will relate to the most appropriate method for detecting and enumerating coliforms and *E. coli* from river water, depending on the degree of accuracy and the rapidity of results which are sought. In addition, the recommendations will also indicate approaches which will allow industry to best deal with produce which have been irrigated with polluted river water, thereby reducing the risk posed to the health of the consumer.

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## CHAPTER 2

### LITERATURE REVIEW

#### 2.1. STATE OF SOUTH AFRICAN WATER RESOURCES

##### 2.1.1. South African water scenario

South Africa is a water scarce country. This reality becomes apparent when the annual average rainfall of 500 mm is compared to the world's 860 mm per annum (Friedrich *et al.*, 2009). This is in conjunction with the fact that South Africa attributes a large part of its gross domestic product (ca. 2.2% in 2007) (Anon., 2008) to agricultural activities. These activities include export fruit and vegetables that require irrigation, which demands that South Africa's water resources – 52% of which is assigned for agriculture (Jackson *et al.*, 2009) – be managed wisely. The primary source of irrigation water for South African crops are rivers, the most readily available and inexpensive form of this resource.

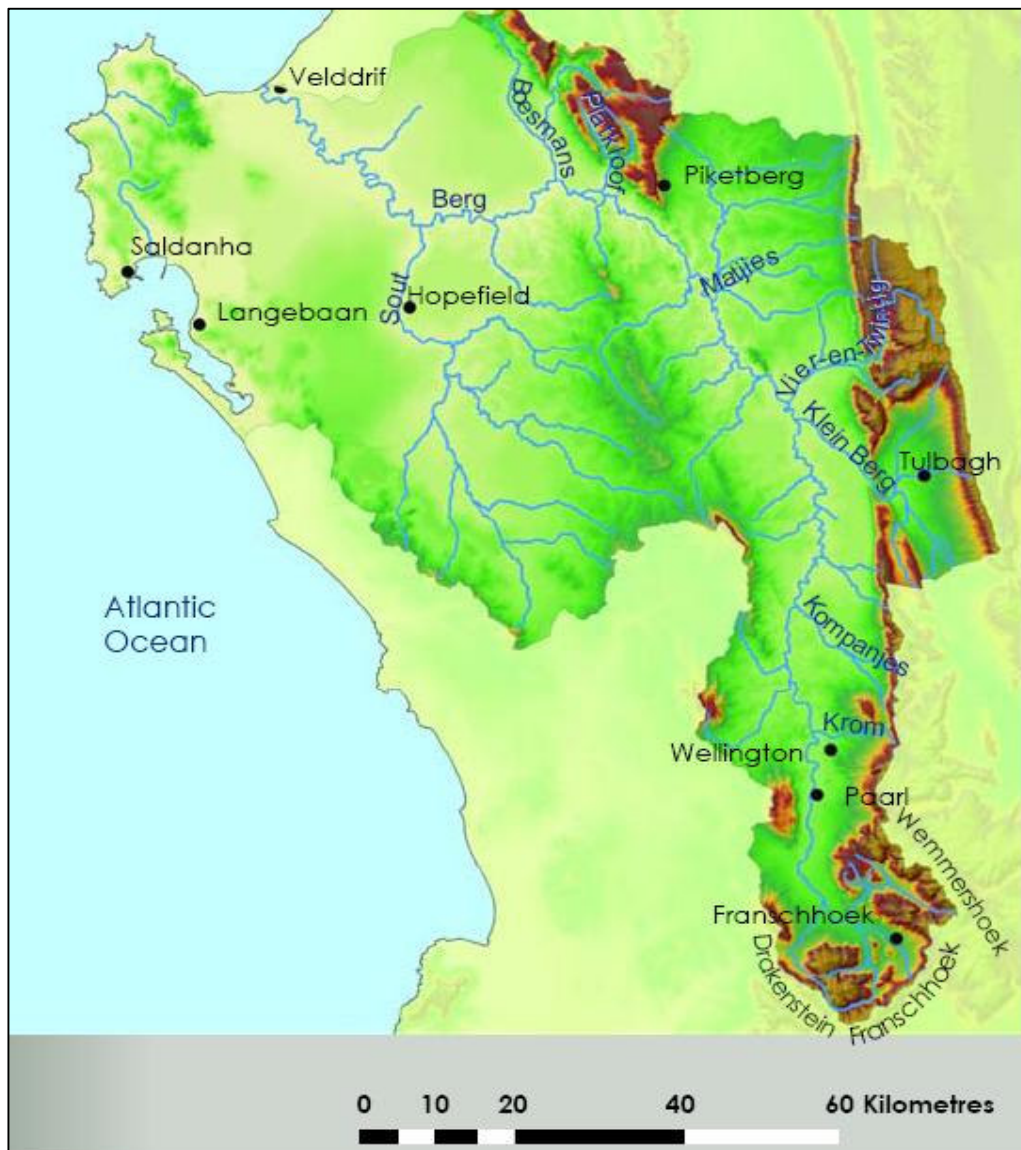
Irrigation, however, is not where the burden of South African rivers ends. River water resources are charged with the task of aiding in the generation of power, providing a place for religious ceremonies, recreational water use and providing towns, cities, mining and industries with water (DWAF, 2004). The 2001 population census showed that 84.4% of households had access to piped water, while the balance utilises resources such as boreholes, rivers and springs. Only a third of households with access to piped water had a tap in the home (Wenhold & Faber, 2009). In 2007, a general household survey (non-census data) indicated that the percentage of urban households with access to piped water had increased to 89.8%, while only 31.2% of rural households had household connections (WHO/UNICEF, 2010). Against the backdrop of a newly emerged democracy, one of the priority development goals of the country is delivery of water and sanitation to all or as government policy states, “*Everybody in South Africa has the right to a basic amount of water and a basic sanitation service...*” (DWAF, 2003). This policy has been implemented through the provision of 6 kL of free water per month per household, and also aims to satisfy the United Nations' Millennium Development Goals of reducing the number of individuals without potable water by 50% by 2015 (Friedrich *et al.*, 2009; Wenhold & Faber, 2009). The policy, however theoretically admirable, is creating problems due to a system of bad prioritisation. The emphasis is placed squarely on the delivery of clean, potable water without focusing on the subsequent hygienic disposal of used water (Jackson *et al.*, 2006). Failure to effectively implement sanitation poses a risk as dangerous substances from sewage or household products can leach into the natural environment (Jackson *et al.*, 2007). In addition, it is a publically known fact that, due to failing and neglected infrastructure, a large number of water treatment plants are either operating inefficiently or, worse, not at all (Dr. J.M. Barnes, Division of Community Health, Faculty of Health Sciences, Stellenbosch University, personal communication, 2008; SAPA, 2011). Power

outages in the country due to problems with the national electricity provider further confounds the situation, since raw sewage simply flows through these sewerage works during power cuts. This sewage contains massive amounts of micro-organisms, unionised ammonia and nitrite combined with a high biological oxygen demand (Finnegan *et al.*, 2009). Farming activities relating to animal husbandry could also serve to contaminate water sources further, as animals carry and shed a variety of enteric pathogens (Brackett, 1999) such as *E. coli* O157:H7 and *Salmonella* which can enter surface water (Beuchat, 2002). All of the aforementioned wastewater, whether from settlements without proper sanitation, animals or failing water treatment plants, eventually finds its way into bodies of surface water (Finnegan *et al.*, 2009) such as rivers. These rivers are, in addition to domestic and recreational use, being used to irrigate crops that may receive minimal postharvest processing or be consumed completely raw. In 1999, Brackett reported that overhead irrigation is very likely to contaminate produce if the irrigation water used contains pathogenic organisms. Their presence should further raise the alarm regarding the state of our rivers as these pathogens will likely be transmitted to humans consuming the produce (DWAF, 1996).

### **2.1.2. Berg River catchment**

The Western Cape is a financially prosperous province owing in part to its agricultural activities. The Berg River catchment in the south-western Cape is one of the most important contributors, and in 2008 a farm gate value of 1.3 billion Rand was amassed for the economy of the country through the production of irrigated products in this region (Louw, 2008). The agricultural activities in the catchment are mainly focused on forestry activities, animal husbandry, grain production, grapes and deciduous fruit. These deciduous fruits comprise a large part of the minimally processed foods (MPFs) which are produced in the Berg River catchment, and include products such as apples, pears, peaches, plums, apricots and citrus (Louw, 2008). In addition, vegetable products which are often consumed raw as MPFs, such as lettuce and tomato, are also grown in this region. For the cultivation of these products, the river is the main source of irrigation water in the Western Cape and flows for 285 km after originating 1 500 m above sea level in the Franschhoek and Drakenstein Mountains, as indicated in Figure 2.1. The figure further shows that the Berg River undulates northward to pass Paarl, Wellington, Hermon and Gouda. After being joined by two of its tributaries, the Klein Berg and Vier-en-Twintig rivers, it proceeds to pass by Porterville and Piketberg before making its way to the coast via Velddrif to discharge into St. Helena Bay (DWAF, 2004).





**Figure 2.1** Geographic location of the Berg River in the Western Cape (extracted from the DWAF State-of-rivers report on the Berg River system (DWAF, 2004))

As a result of the population increase in the Western Cape and subsequent increase in water demand, plus increasingly dry summers and severe water restrictions (DWAF, 2004), the Berg Water Project (BWP) was implemented in May 2002 with a 1.8 billion Rand budget (DWAF, 2004). The aim of the project was to construct a 65 m high dam wall, thereby creating a dam (130.1 million cubic litres of water) 990 m in breadth and 220 m in width (Rossouw & Grobler, 2008), 5 km outside Franschhoek. As part of the process an abstraction works below the Dwars River confluence was to be implemented, which by 2007 would increase the yield of the Berg-Riversonderend Scheme to 523 million cubic meters per annum (DWAF, 2004). The Dam was

also designed to be able to release 200 m<sup>3</sup>/s of water in peak release to create flood releases for the correct functioning of the ecosystem (Rossouw & Grobler, 2008).

The Berg River Dam was completed in 2007, and by July 2007 had started storing water. Due to an exceptionally good winter, the Dam was filled to capacity by the winter of 2008 (City of Cape Town Communication Department, 2009) and has since been in use to ensure a steady supply of water in the catchment.

The Berg River drains an area of around 8 980 km<sup>2</sup> (DWAF, 2004), with numerous informal settlements and improperly functioning sewerage treatment plants resulting in large amounts of pollution entering the system. This manifests as microbiological contamination of the water with organisms either occurring in a free planktonic state, attached to rocks or solid pollution in a biofilm (Jackson *et al.*, 2007) or attached or embedded within particles (Cantwell & Hofmann, 2008).

The repercussions of the state of the Berg River's water have already begun manifesting, and will hit the country hard if the problem is not addressed. In 2005 the European Union (EU) warned South African export farmers that the extent of pollution of the Berg River is running them the risk of rejection of their produce, since water from this important system is used to irrigate their fruit (Anon., 2005). This warning was based on results indicating that the *Escherichia coli* levels in the Berg River exceeds the maximum amount prescribed by the EU by as many as 2400 times. As the headline "Berg River endangering health of the economy" so ominously states, the economic implications of having export fruit rejected by Europe carries significant far-reaching implications for the country. Jackson and co-workers (2007) also observed recently that the pollution levels in the Berg River are increasing. Furthermore, another important and potentially life-threatening concern emerges from these reports: if water from the Berg River is inappropriate for irrigation; how can it be appropriate for direct human consumption and what are the associated health implications?

The findings related to South African water quality and subsequent newspaper headlines are often dismissed as "sensationalist" or "alarmist" by those bearing responsibility for the state of South African waters. This attitude is problematic for improving the situation, as commitment to finding solutions must be preceded by determination of the extent, and acknowledgment, of the problem. An investigation into the extent of the problem will be inextricably linked to the guideline values set out by the Government to ensure the safe utilisation of water, but what are the South African regulations regarding the levels of *E. coli* or faecal coliforms in our water?

This question is not one that can be readily answered: it is only after an extensive search of literature that regulatory guidelines for South African water were obtained from the National Microbial Monitoring Programme (NMMP) of the Department of Water Affairs and Forestry (DWAF), known as the Department of Water Affairs (DWA) since 2009. As indicated in Table 2.1, these guidelines recommend that >10 faecal coliforms per 100 mL of untreated water should be regarded as a risk for consumption, while a level of faecal coliforms higher than 4 000 per 100 mL should be assessed as a potential risk for irrigating crops that are eaten raw (DWAF, 2008). It



must also be stressed that these values are only guidelines, and that no official standards could be found for South Africa's irrigation water.

**Table 2.1** Guidelines for assessing the potential health risk for the four water uses (DWAF, 2008)

<b>Sensitive Water Use</b>	<b>Guideline (faecal coliforms.100 mL<sup>-1</sup>)</b>
Drinking untreated water	>10
Full or partial contact	>2 000
Irrigation of crops eaten raw	>4 000
Drinking after limited treatment	>20 000

These guidelines do not correspond to the quality guidelines of the World Health Organization (WHO), which recommends that water used for the irrigation of crops that are likely to be eaten raw should not have more than 1 000 faecal coliforms per 100 mL (WHO, 1989). While the cut-off values indicated in Table 2.1 were not intended to replace the target water quality ranges set by DWAF in 1996, the South African Government are using these relaxed values for water use risk assessments as part of the NMMP. For example, DWAF's 1996 limit for irrigation water was set at 2 000 faecal coliforms per 100 mL, but it is clear from Table 2.1 that the NMMP would only register an associated risk with this water use at a level above 4 000 faecal coliforms per 100 mL.

As for the question regarding the state of our waters, the answer is no less worrying. In 2003, Dr Jo Barnes had already shown that the Plankenburg river system in Stellenbosch is heavily polluted and that consequent health risks would emerge from this state of affairs. The data shown in Table 2.2 were obtained during the period of 1998 to 2002, and indicates the state of the river as it was a decade ago.

**Table 2.2** Faecal coliforms detected in the Plankenburg River at points above and below Kayamandi (cfu.100 mL<sup>-1</sup>) (Barnes, 2003)

<b>Sampling date</b>	<b>Above Kayamandi</b>	<b>Below Kayamandi</b>
May 1998	12 000 <sup>a b</sup>	16 000 <sup>a b</sup>
August 1998	329	172 300 <sup>a b</sup>
December 1998	6 310 <sup>a b</sup>	792 000 <sup>a b</sup>
January 1999	347	493 000 <sup>a b</sup>
June 1999	10 860 <sup>a b</sup>	49 300 <sup>a b</sup>
December 1999	329	4 930 000 <sup>a b</sup>

**Table 2.2 continued**

**Table 2.2 continued**

<b>Sampling date</b>	<b>Above Kayamandi</b>	<b>Below Kayamandi</b>
January 2000	130	17 420 000 <sup>a b</sup>
June 2000	493	2 640 <sup>a b</sup>
December 2000	493	3 290 000 <sup>a b</sup>
January 2001	3 290 <sup>a</sup>	3 290 000 <sup>a b</sup>
July 2001	278	32 900 <sup>a b</sup>
December 2001	221	69 900 <sup>a b</sup>
January 2002	493	17 500 <sup>a b</sup>
June 2002	3 454 <sup>a</sup>	493 000 <sup>a b</sup>
October 2002	1 300 <sup>a</sup>	129 000 <sup>a b</sup>

<sup>a</sup> Faecal coliform level in violation of WHO guidelines for irrigation water

<sup>b</sup> Faecal coliform level in violation of DWAF guidelines for irrigation water

Interesting observations can be made based on these results, like the increase in the number of faecal coliforms downstream from Kayamandi. The most important to note, however, is that nearly all the values in the table exceed the limit for irrigation water as set by the WHO (WHO, 1989). In fact, most values do not only exceed this value, but also the potential risk value as defined by the DWAF (DWAF, 2008). Except for the clear violation of both local and international guidelines regarding our water, the extent of the transgression should be noted at a point such as the January 2000 value for the water below Kayamandi, with a staggering 17 420 000 faecal coliforms per 100 mL.

In more recent and ongoing work done at Stellenbosch University in collaboration with the Universities of Pretoria, KwaZulu Natal and Venda, more data were generated in the support of the earlier work done by Barnes. As shown in Table 2.3, data for the Plankenburg River below Kayamandi during the sampling period of 2007 to mid-2008 showed the presence of known and potential pathogens such as *Salmonella*, *Listeria*, coagulase-positive staphylococci and intestinal enterococci. This is troubling since some of these organisms can cause disease in human hosts at very low infective doses (see Table 2.4 later). In addition, high levels of faecal coliforms, indicative of pollution with faecal matter, were also detected.

From the data it is clear that over the last ten years, there has been no improvement in the quality of the water in this river, both in numbers and transgression of regulations, and that governing bodies are not paying heed to the numerous warning signs regarding the state of South African river water.

**Table 2.3** Microbiological data obtained from the Plankenburg River below Kayamandi (September 2007 to April 2008) (Ackermann, 2010; Lotter, 2010)

	September 2007	October 2007	November 2007	February 2008	March 2008	April 2008
Faecal coliforms (cfu.100 mL <sup>-1</sup> )	14 000	17 000	160 000	28 000	35 000	490
<i>Salmonella</i>	TG	TG	TG	ND	TG	TG
<i>Listeria</i>	-	TG	TG	TG	TG	TG
Staphylococci (cfu.mL <sup>-1</sup> )	80	TNTC	ND	390	ND	0
Enterococci (cfu.100 mL <sup>-1</sup> )	20	5	TNTC	65	8	43

TG - typical growth (*Salmonella*: clear with black centers on XLD agar; *Listeria*: olive green with black centers on Oxford agar)

ND - none detected

TNTC - too numerous to count

If the microbial contamination of these rivers continues to worsen it would mean a severe human health risk, especially to those individuals using the river for drinking, washing and recreational purposes. Additionally, it would increase the risk of disease for persons consuming food irrigated with this water and could subsequently lead to the loss of export licences. The aforementioned detrimental factors will in turn increase the country's burden of disease and result in a further economic downturn.

## 2.2. IMPACT OF CONTAMINATED RIVER WATER ON HUMAN HEALTH

### 2.2.1. Direct impact

#### Water to health

The data in Tables 2.2 and 2.3 confirm that there is a contamination problem with South African river water, but the question to be asked is why is the exceeding of guidelines problematic? Human health standards for faecal indicator bacteria in drinking, recreational and shellfish waters are based on epidemiological studies (Field & Samadpour, 2007). Therefore, the problem of polluted river water lies in the concept of infective doses, which indicate numbers of organisms in food or water which have become high enough to infect healthy individuals (see Table 2.4).

**Table 2.4** Orally transmitted waterborne pathogens and their significance in water supplies (WHO, 2006)

Pathogen	Health significance	Persistence in water supplies <sup>a</sup>	Resistance to chlorine <sup>b</sup>	Relative infectivity <sup>c</sup>
<b>Bacteria:</b>				
Enterohaemorrhagic <i>E. coli</i>	High	Moderate	Low	High
Pathogenic <i>E. coli</i> <sup>d</sup>	High	Moderate	Low	Low
<i>Salmonella typhi</i>	High	Moderate	Low	High
Other salmonellae	High	May multiply	Low	Low
<i>Shigella</i> ssp.	High	Short	Low	Moderate
<b>Viruses:</b>				
Hepatitis A	High	Long	Moderate	High
Noroviruses and saproviruses	High	Long	Moderate	High
Rotavirus	High	Long	Moderate	High

<sup>a</sup> Period detected in water at 20 °C – Short: 1 week, Moderate: 1 week to 1 month, Long: >1 month.

<sup>b</sup> In water treated at conventional chlorine doses and contact times – Moderate resistance: pathogens not completely destroyed.

<sup>c</sup> Dose required to cause infection in 50% of healthy adult humans.

<sup>d</sup> Including enteroinvasive, enteropathogenic and enterotoxigenic strains.

For the enteric bacteria, this infective dose value tends to lie between 10 and 1000 (DWAF, 1996; Barnes, 2003), while it could be as low as 10 cells for enterohaemorrhagic *E. coli* (EHEC) (Schmid-Hempel & Frank, 2007) and one infective unit for some viruses (DWAF, 1996). For *E. coli* O157:H7, disease can be caused at levels as low as 100 cfu.g<sup>-1</sup> (Velázquez *et al.*, 2009), depending on the virulence of the pathogen and the immunity of the host (Barnes, 2003). Especially in the South African context, with millions of people infected with human immunodeficiency virus (HIV) and in various stages of acquired immunodeficiency syndrome (AIDS) or related immunodeficient illnesses such as tuberculosis, the immunity of the host is of enormous importance to the infective dose level. From the data given in Tables 2.2 and 2.3, along with the health significance and infective doses given in Table 2.4, it is clear that contact with water from the Plankenburg River is a potential threat to human health.

In the global context, nearly half of the hospital beds in the world are occupied by patients affected by diseases relating to water quality (Wenhold & Faber, 2009). Water-associated illness contributes significantly to the global burden of disease (Wenhold & Faber, 2009) at around 5.7% (Shannon *et al.*, 2007) or 1.5 billion people at any given time (Wilkes *et al.*, 2009). This is caused by chemical contamination with pesticides and disinfectants or by microbiological contamination which encompasses bacterial, viral and parasitic organisms present (Wenhold & Faber, 2009). It is

this microbiological contamination which is mainly responsible for diarrhoeal disease contributing 2.2 million of the 3.4 million deaths per year related to water-associated illness (Wenhold & Faber, 2009). This is in part a consequence of 2.6 billion people in the world who are still living without adequate sanitation (Maïga *et al.*, 2009). The data given in Table 2.5 shows various categories of infectious diseases associated with water, and their routes of transmission.

**Table 2.5** Infectious diseases associated with water (Wenhold & Faber, 2009)

Transmission route	Description	Examples of infections
Waterborne	Water acts as a passive vehicle for the infective agent, thus transmission is via consumption of contaminated water	<ul style="list-style-type: none"> <li>Bacterial infections - <i>Salmonella</i> typhoid, enterobacteria, cholera</li> <li>Viral infections - hepatitis A, rotavirus</li> <li>Parasitic infections - amoebiasis, giardiasis, intestinal protozoa, ascariasis, hookworm</li> </ul>
Water-washed	Infections as a result of insufficient quantities of water for personal or domestic hygiene	<ul style="list-style-type: none"> <li>Enteric infections - certain diarrhoeal diseases and gastroenteritis</li> <li>Skin infections - scabies</li> <li>Lice-borne infections - typhus</li> <li>Eye and ear infections - otitis, conjunctivitis, trachoma</li> </ul>
Water-based	A necessary part of the life cycle of the infective agent takes place in an aquatic organism; thus infection is transmitted through repeated contact with or ingestion of contaminated water e.g. bathing or washing clothes	<ul style="list-style-type: none"> <li>Infections caused by crustaceans - guinea worm disease</li> <li>Infections caused by fish - diphyllbothriasis</li> <li>Infections caused by shellfish - flukes, schistosomiasis (bilharzias)</li> </ul>
Water-related (vector-borne)	Infections spread by insects that breed in water or bite near it	<ul style="list-style-type: none"> <li>Infections caused by mosquitoes - malaria, yellow fever, haemorrhagic fever</li> <li>Infections caused by tsetse flies - trypanosomiasis</li> <li>Infections caused by blackflies – onchocerciasis</li> </ul>

Of the categories of water-associated diseases as given in Table 2.5, only waterborne and water-washed is of significance to this section and in fact the overall literature study due to their relation to socio-economic and water management problems.

## Microbes in river water

The consumption of river water contaminated with waterborne pathogens as those shown in Table 2.4 manifests in a variety of disease symptoms. The degree of illness is dependent on factors such as the age, gender, physiological state and immune status of the host (Krämer *et al.*, 2010) as well as the pathogenicity of the organism (Barnes, 2003), but many of these pathogens are capable of being lethal to both compromised and healthy humans. Of these pathogens, *E. coli* is of particular importance since it is not only a pathogen of interest for water microbiology, but also an indicator of faecal pollution (Jagals *et al.*, 2006) in water as well as food.

*Escherichia coli* can be subdivided into four groups based on phylogenetics namely A, B1, B2 and D, with groups B2 and D comprising the pathogenic strains responsible for extraintestinal infections. These strains can be divided into meningitis-associated *E. coli* (MNEC) and uropathogenic *E. coli* (UPEC) and are responsible for haemolytic uremic syndrome (HUS) (Orsi *et al.*, 2008), typified by haemolytic anaemia and acute renal failure manifesting in 2 to 7% of cases (WHO, 2011); newborn meningitis, urinary tract infections and sepsis.

Groups A and B1 are allocated to the intestinal pathogen strains of *E. coli* namely diffusely adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC) (Orsi *et al.*, 2008). In terms surface water contamination, five of these strains are implicated namely EAEC, EHEC, EIEC, EPEC and ETEC (Jagals *et al.*, 2006). Disease related to the presence of intestinal pathogenic *Escherichia coli* strains in the water include symptoms such as diarrhoea, as well as abdominal cramps, headaches and nausea (WHO, 2011).

More specifically, EAEC strains are usually associated with persistent non-bloody diarrhoea in toddlers by action of adhering to the intestinal mucosa, and do not produce either heat labile nor heat stable enterotoxigenic toxins but rather *Shigella* enterotoxin 1 (chromosomally coded) and heat labile enteroaggregative ST toxin (plasmid-coded) (Jagals *et al.*, 2006).

EHEC is categorised with the subgroup of *E. coli* known as Shiga-toxin producing *E. coli* (STEC) which are typified by their production of verocytotoxins. EHEC specifically is able to produce Shiga toxin 1 and Shiga toxin 2, with Shiga toxin 1 differing from the toxin produced by *Shigella dysenteriae* by only a single amino acid (Jagals *et al.*, 2006). The mode of function of the Shiga toxins is to prevent protein synthesis within the host cells which results in cell death (Ram *et al.*, 2009).

This strain is responsible for haemorrhagic colitis (Ram *et al.*, 2009), haemolytic uremic syndrome (Ram *et al.*, 2009), non-bloody diarrhoea (Jagals *et al.*, 2006) and even death (Ram *et al.*, 2009). It has also been associated with outbreaks related to contaminated water, whilst enterohaemorrhagic *E. coli* O157:H7 have frequently been linked to food related outbreaks (Jagals *et al.*, 2006).

EIEC, like *Shigella*, is responsible for bacillary dysentery through an action of invading epithelial cells of the intestine; multiplying, spreading and leaving massive cell destruction in its

wake, but is differentiated from *Shigella* based on the lack of Shiga toxin production (Jagals *et al.*, 2006). EPEC and ETEC are both causative agents of diarrhoea, but while EPEC is the cause of almost exclusively infant diarrhoea ETEC causes diarrhoea in children as well (Jagals *et al.*, 2006), mainly due to the consumption of food and water that has been contaminated (Ram *et al.*, 2009). EPEC does not produce Shiga, heat labile or heat stable toxins but does, however, produce heat-stable enterotoxin or cyto-lethal distending toxin. ETEC on the other hand, produces heat labile or heat stable toxins. The heat labile toxins are subdivided under LT1 and LTII (Jagals *et al.*, 2006). LTII is produced in animal-specific strains, while LT1 is highly similar, in terms of structure and function, to the cholera toxin (Jagals *et al.*, 2006). The virulence of EPEC strains is determined by LT1 and ST1 genes (Ram *et al.*, 2009).

Consumption of water contaminated with *Salmonella* can lead to gastroenteritis, bacteraemia or septicaemia and the typhoid or enteric fever. The species responsible for enteric illness can be categorised as either the typhoidal species or serovars (*Salmonella typhi* and *S.paratyphi*) or non-typhoidal species. In non-typhoidal cases, the diarrhoea lasts for 3 to 5 days with accompanying fever and abdominal pain. Typhoid fever is much more severe and is potentially life-threatening.

The *Shigella* species are responsible for intestinal diseases such as bacillary dysentery, and for up to 600 000 deaths a year. *Shigella sonnei* infection usually results in mild illness, whilst *Shigella dysenteriae* produces Shiga toxin and results in more serious illness (WHO, 2011). According to the WHO, these organisms can lead to infection at a level of 10 to 100 organisms, and seem to be very well adapted to causing human disease.

In terms of the viral pathogens, the presence of Hepatitis A in the water will lead to infectious hepatitis which describes liver damage and an inability of the liver to remove bilirubin from the blood, resulting in jaundice and dark urine (WHO, 2011). Other symptoms include fever, malaise and stomach cramps; and in children nausea, vomiting and diarrhoea is more prevalent than in adults (Barnes & Taylor, 2004). The rotaviruses are the greatest cause of infant mortality in the world. Rotaviruses infect the small intestinal villi, which leads to the disruption of sodium and glucose transport, with symptoms such as diarrhoea, fever, abdominal pain, dehydration and metabolic acidosis resulting (WHO, 2011). The transfer of noroviruses is usually through the oral-faecal route, and these viruses are responsible for gastroenteritis when they are consumed with contaminated water (Prof M.B. Taylor, Department of Virology, Pretoria University, personal communication, 2009).

### **2.2.2. Indirect impact**

The impact of irrigating produce that will be eaten raw with water contaminated with pathogens could be devastating not only to an individual's health, but also to the economy of the country of the outbreak and, in export scenarios, the country of origin. Lu *et al.* (2004) stated that agricultural products irrigated with water containing pathogens risk becoming contaminated with pathogens. In

support of this statement, Barnes and Taylor (2004) reported that fruit and vegetables can be contaminated with wastewater used for irrigation, and that viral contamination usually occurs prior to harvesting in produce with a short growth period. Also, there has been a steady increase in the per capita consumption of raw fruits and vegetables (Brackett, 1999) due to an increasing aversion for the consumption of animal products (Beuchat, 1996). In addition, the media and public health institutions also encourage the consumption of at least five servings of vegetables and fruit per day, due to their important function in the human diet and subsequent health benefits (Abadias *et al.*, 2008). Canadian Statistics (Anon., 2009) revealed that 43.7% of Canadians consumed five or more fruit and vegetables a day in 2008, in comparison with only 37.6% in 2001. Unfortunately as the consumption of these products increases, so does their importance as a vehicle of foodborne illness increases (Brackett, 1999).

Hurst (1995) foresaw a number of problems with minimally processed foods (MPFs), such as the risk of having no step where pathogenic organism numbers are reduced, as many pathogens are able to proliferate even under refrigeration conditions. If refrigeration conditions are not maintained throughout, a temperature of 7°C or higher could encourage the growth of pathogens such as *Salmonella*, *Staphylococcus*, *Clostridium botulinum* and *Bacillus*. Also, during washing and trimming of the produce, the naturally occurring microbes on the product would be removed and subsequently give pathogens a competitive advantage (Hurst, 1995). In such a case the numbers of pathogens could increase sufficiently to infect an individual. Furthermore, modified atmosphere may deter the growth of some pathogens but may create an environment in which pathogens such as *Listeria monocytogenes* thrive (Hurst, 1995).

A little more than a decade after Hurst's predictions, fresh fruit and vegetables are becoming notorious vehicles for the transmission of pathogens (Abadias *et al.*, 2008). According to Da Cruz (2005), these products were responsible for a staggering 23% of food-related outbreaks in the period from 1990 to 2005 and in 2008, fresh produce had become the second largest contributor of food-related illness in the United States with around 73 000 *E. coli* O157:H7 and two million salmonellosis infections each year (Bialka & Demirci, 2008). An increase in the number of detected outbreaks of disease associated with the consumption raw fruits and vegetables (Abadias *et al.*, 2008; Velázquez *et al.*, 2009) have been observed since the 1980's (Burnett & Beuchat, 2002). These increased numbers can primarily be attributed to improvements in epidemiological surveillance and not necessarily a true increase in the number of outbreaks. However, changes in consumption patterns due to the health benefits of fruit and vegetables (Selma *et al.*, 2007; Abadias *et al.*, 2008), changes in the production of fruit and vegetables and the emergence of "new" pathogens not traditionally associated with fresh produce eaten raw (Beuchat, 2002) have contributed to a true increase in the number of outbreaks. Large outbreaks that were well covered in the media involved alfalfa sprouts, cantaloupes, lettuce and tomatoes (Brackett, 1999).

The Centers for Disease Control and Prevention (CDC) estimate that approximately 76 million Americans suffer from foodborne illness every year, with 300 000 of them being hospitalised



and 5 000 eventually dying (King, 2006). Of these cases, most can be attributed to bacteria, viruses and parasites occurring on the food (King, 2006). While many varieties of fresh produce have been implicated, sprouts are a product of particular concern since there is a possibility for pathogens to grow during sprouting (Abadias *et al.*, 2008) as the environmental requirements for sprouting is similar to those for bacterial growth, and these products have therefore been recognised for the last decade as a major vehicle for foodborne illness (Johnston *et al.*, 2005). In 1996 Japan saw over 6 000 culture-confirmed *E. coli* O157:H7 cases in one of the largest outbreaks connected to sprouts, in this case the consumption of radish sprouts (Johnston *et al.*, 2005). *Listeria monocytogenes* is implicated in 43% of all deaths associated with foodborne infection in the United States between 1998 and 2002, several of these being the related to the consumption of fresh produce that was contaminated, quite possibly by sheep faeces in the soil where it had been grown (Dreux *et al.*, 2007). Non-virulent isolates of *Yersinia enterocolitica* have been isolated from horticultural products, ready-to-eat vegetables and lettuce in many countries including Italy, Korea, Norway and Australia, but in 2005 in Japan, a severe outbreak of *Y. enterocolitica* O:8, a highly pathogenic serotype, on salads was reported (Velázquez *et al.*, 2009). In Argentina, the first case of foodborne illness linked to MPFs was in March 2006, with ready-to-eat salads contaminated with *E. coli* O157:H7 and *Staphylococcus aureus* spp. *aureus* being implicated (Velázquez *et al.*, 2009). Another case which received worldwide media attention was in September 2006, when Wisconsin which saw 102 hospitalisations, 31 cases of HUS and three deaths related to the consumption of uncooked fresh bagged spinach (King, 2006; Abadias *et al.*, 2008). After culturing of leftover spinach by the CDC, Food and Drug Administration (FDA) and state public health laboratories, *E. coli* O157:H7 was implicated. This was the first outbreak of its kind associated with spinach, but lettuce has been implicated numerous times with twenty *E. coli* O157:H7 outbreaks caused by leafy greens (King, 2006; Velázquez, 2009). In 2011, one of the worst known foodborne outbreaks occurred in northern Germany. Fenugreek seeds from Egypt were eventually implicated as the vehicles of the outbreak, which was caused by a new pathogenic strain of *E. coli* named enteroaggregative-haemorrhagic *E. coli* (EAHEC). The outbreak caused 3 368 individuals to fall ill, and caused 36 fatalities (Brzuszkiewicz *et al.*, 2011).

The consumption of fruit juice that has not been subjected to a heat treatment has also been implicated in several cases of food-related illness, with four outbreaks of *E. coli* O157:H7 in the 1990's epidemiologically associated with unpasteurised apple juice. A few years later, in 1999, 300 people fell ill with salmonellosis after consuming unpasteurised orange juice (Kenney & Beuchat, 2002).

The organisms occurring on produce that are of particular concern, apart from viruses, include *Bacillus cereus*, *Clostridium botulinum*, EPEC, ETEC and EHEC, *Campylobacter* spp., *Listeria monocytogenes*, *Shigella* spp., *Salmonella*, *Yersinia enterocolitica* and parasites such as *Giardia*, *Cyclospora* and *Cryptosporidium* (Beuchat, 2002; Abadias *et al.*, 2008; Velázquez *et al.*,

2009), with *E. coli* O157:H7 and *Salmonella* being the most prominent culprits of gastroenteritis in North America (Johnson *et al.*, 2003).

The increase in the number of food-related outbreaks relating specifically to fresh fruit and vegetables that are consumed raw is attributed to changes in the production of these foods to meet the demands of an ever increasing population while keeping production costs as low as possible. Prior to harvest this is achieved by using uncomposted manure (Selma *et al.*, 2007) instead of chemical fertiliser but more importantly, using reclaimed water for irrigation (Harwood *et al.*, 2005) – water which contain pathogens (Beuchat, 2002) – instead of municipal or purified water which would be more costly by several orders of magnitude. The irrigation of produce with wastewater should be managed very prudently as improperly or inadequately treated wastewater may contain pathogens (Selma *et al.*, 2007), and this goes for any water that is potentially contaminated with food-associated pathogens. Brackett (1999) also observed that cross contamination with manure from domestic and wild animals as well as contact with contaminated water posed a risk, and suggested that only clean potable water be used for irrigation after planting. McLain *et al.* stated in 2008 that reclaimed wastewater is increasingly being used as a source of irrigation water and stressed the importance of accurate monitoring of the water quality to ensure there is no risk involved in human contact, a point which is certainly equally important when using contaminated river water for irrigation.

## **2.3. PERSISTENCE OF PATHOGENS IN THE ENVIRONMENT**

### **2.3.1. Survival in water systems**

Another important factor to keep in mind is the survival of the aforementioned pathogens in the environment, as well as their survival time. It is known that pathogens will lose their viability and decay at an exponential rate once they are outside their host (Barnes, 2003). However, the persistence of these organisms can be influenced by a number of factors, the most important being the temperature of their environment. In water, a higher temperature and the effects of ultraviolet radiation from the sun will accelerate the decay rate and inactivation of all organisms (Curtis *et al.*, 1992; Maïga *et al.*, 2009). In contrast, a fairly high concentration of biodegradable organic carbon can sustain these organisms and even facilitate their multiplication. Dissolved organic matter can also serve to attenuate the UV range of the solar spectrum (Maïga *et al.*, 2009). Other factors that play a role in the persistence of bacteria in water include the type of organism, the pH and salinity of the water, the presence of toxic substances and algae in the water (Curtis *et al.*, 1992) and competition and predation (DWAF, 1996). Fortunately, viruses are unable to multiply in the environment (Curtis *et al.*, 2000). They are, however, still a major source of concern since it may take only one viable unit to cause infection (DWAF, 1996). In 1980, Dutka and Kwan showed that

*E. coli* placed in membrane filter chambers were able to survive in Lake Ontario for a minimum of 28 days. Similarly, Sadovskii and co-workers (1978) showed that drug-resistant *E. coli* strains were able to survive in irrigation pipes for a minimum of eight days, and for at least 18 days in soil. It has also been proven that *L. monocytogenes* can persist in soil for up to 295 days (Beuchat, 1996), and *Salmonella enterica* (Natvig *et al.*, 2002; Islam *et al.*, 2004b) and *E. coli* O157:H7 (Islam *et al.*, 2004a) survive for 100 to 250 days in soil fertilised with manure and subsequently on the produce grown in the soil. The introduction of *E. coli* to filtered and autoclaved river water showed that there was no loss of viability for up to 260 days (Flint, 1987). However, the survival times were shortened when the water was not autoclaved and therefore contained natural background organisms which could compete for nutrients (Flint, 1987). It has also been demonstrated that pathogens such as *L. monocytogenes* and *Salmonella* could survive in sewage sludge applied as an agricultural soil amendment for months (Brackett, 1999). Work done by Oragui & Mara in 1983 showed that the *E. coli* and faecal streptococci in fresh water disappeared after five weeks, but that these indicator organisms survived for up to 26 weeks at 20°C in normal sewage. Boyle and co-workers (1991) reported that *Staphylococcus aureus* did not survive for a week in distilled water incubated at 10°C, 25°C and 37°C; but that *Pseudomonas aeruginosa* survived for up to five months at all these temperatures.

Sunlight damage from radiation on water surfaces manifests itself either as photobiological (damage to the microbe DNA) or as photo-oxidative (oxidation of cellular components) damage (Maïga *et al.*, 2009). Schultz-Fademrecht and co-workers (2008) showed that the level of culturable faecal bacteria in river water was dramatically decreased with increased intensity of sunlight radiation. These workers reported that  $I_{(290-390\text{nm})}=40 \text{ W.m}^{-2}$  of sunlight radiation reduced the faecal coliform and intestinal enterococci populations in river water with pre-treated sewage water by 50% within an hour. Additionally, the work showed that faecal coliforms have a higher half-life when compared to enterococci at a sunlight intensity of  $I_{(290-390\text{nm})}=8 \text{ W.m}^{-2}$  (Schultz-Fademrecht *et al.*, 2008).

The damage inflicted by sunlight on the microbial cell is dependent on several factors such as oxygen, pH and the presence of humic substances. In water with a high concentration of oxygen, cellular chemicals (such as porphyrin or flavin) are elevated to an excited state by sunlight and can react to form toxic oxygen species such as hydrogen peroxide, hydroxyl radicals, singlet oxygen and superoxide (Curtis *et al.*, 1992). Chemicals outside the cell can also enter an excited state and react with oxygen; these are named humic substances and comprise photosynthetic pigment and organic matter in a refractory and dissolved state. Finally, an increase in pH has been reported to have synergy with the effect of sunlight and increase the extent of sunlight damage (Curtis *et al.*, 1992). However, *E. coli*, like many other microorganisms, has the ability to repair themselves after damage by sunlight or ultraviolet light by either photoreactivation or dark repair (Guo *et al.*, 2009; Maïga *et al.*, 2009). Photoreactivation is facilitated by photolase, an enzyme which repairs UV-induced damage of the DNA through utilisation of 310-480 nm near-UV

light (Guo *et al.*, 2009). In wastewater treated with low-pressure UV light, *E. coli* underwent a 1.2 log photoreactivation after three hours of sunlight exposure (Guo *et al.*, 2009). In addition, research by Cantwell & Hoffman (2008) showed that particles naturally occurring in surface water can protect coliforms and *E. coli* from low-pressure UV light treatments commonly employed at water treatment plants. However, Guo and colleagues (2009) report that medium-pressure UV lamps reduce the ability of the organism to photoreactivate more than low-pressure UV lamps at a dose of 5 mJ.cm<sup>-2</sup> and found subsequent colony forming ability of 32% and 60%, respectively. This advantage of medium-pressure over low-pressure UV is neutralised at an elevated dose of 15 mJ.cm<sup>-2</sup>, where no photoreactivation occurs for either treatment (Guo *et al.*, 2009).

It is also known that during adverse environmental conditions several strains of *E. coli*, such as *E. coli* K-12 (Danese *et al.*, 2000) and *E. coli* O157:H7 (Chen *et al.*, 2004), are able to produce exopolysaccharides (EPS) made up of colanic acid subunits, and this has been suggested to aid in the protection of cells. The EPS play a role in the formation of biofilms that can typically be seen on the surface of polluted water. This happens through a mechanism of the production of a conditioning film for cell adhesion followed by growth and EPS production of the adherent cells. These biofilms are able to facilitate the concentration of pathogens in wastewater (Boyle *et al.*, 1991). Also, when found on foods, they can increase the resistance of cells to removal or inactivation by sanitisers (Ryu & Beuchat, 2004). Schultz-Fademrecht *et al.* (2008) showed that the numbers of faecal coliforms and intestinal enterococci occurring in biofilms on river stones were decreased with sunlight exposure. Faecal coliform numbers in this biofilm without sunlight exposure, however, increased with a factor 1.5 and 3.1 for previous exposure to 40 W.m<sup>-2</sup> and 8 W.m<sup>-2</sup>, respectively. Intestinal enterococci in the biofilm continued to decrease with no sunlight exposure, but at a much lower rate than observed with sunlight exposure (Schultz-Fademrecht *et al.*, 2008).

### 2.3.2. Survival on fresh produce

Produce can become infected through a variety of means during the pre- and post-harvest steps of the production process. The most important sources of pre-harvest contamination are soil, faeces in the environment and contaminated irrigation water (Beuchat, 2002). Post-harvest contamination usually occurs via faeces, human handlers and contaminated harvesting equipment, but can also occur through unconventional vectors such as fruit flies, which have been shown to contaminate apple wounds with *E. coli* O157:H7 (Janisiewicz *et al.*, 1999). The organism could be detected on the apples 48 h after exposure to the fruit flies.

The composition of organisms that are found on a particular food product will depend on one or more factors. These include the surface of the product, which determines the ease with which organisms adhere, the circumstances under which it has been grown (including the irrigation practices), and the inherent properties of the product such as a low pH, protective cuticle, the epithelial structure and the presence or absence of natural antimicrobials (Beuchat, 2002).

Generally speaking, psychrotrophic Gram-negative rods are more prevalent on raw or lightly processed vegetables (Hurst, 1995) while moulds and weakly fermentative yeasts dominate on raw fruits due to the inherently low pH, usually below 4.0, of fruit tissue (Splittstoesser, 1987). However, Beuchat (2002) reported that some pathogenic bacteria are able to adapt to stress conditions, which is conducive to their survival and growth on fruit and vegetables, for example, *E. coli* O157:H7 and *Salmonella* are able to adapt to a reduced pH (Kenney & Beuchat, 2002). Furthermore, yeasts and moulds would ordinarily only decrease the organoleptic quality of the produce and are rarely pathogenic. Nevertheless, their utilisation and subsequent reduction of organic acids present in fruit will increase the pH which could lead to favourable conditions for the growth of *Clostridium botulinum* and the production of its toxin (Beuchat, 2002). If it is possible for *C. botulinum* to grow under these conditions, other pathogens may also be able to grow. It has been found that *E. coli* O157:H7 could still be detected on bovine manure-contaminated lettuce after 15 days holding at 4°C, even at an initial concentration of  $10^0$ - $10^1$  cfu.g<sup>-1</sup> (Beuchat, 2002).

*L. monocytogenes* has been reported as being able to survive on plant material for ten to twelve years (Beuchat, 1996). This bacterium is widely known to grow on various vegetables at refrigeration temperatures with many cases of human listeriosis linked to the consumption of raw vegetables that had come into contact with some form of ruminant manure, be it through soil or irrigation water (Beuchat, 2002). Experiments performed by Beuchat *et al.* (1986) revealed that *L. monocytogenes* numbers increased from  $1.6 \times 10^4$  to  $2.6 \times 10^8$  on raw cabbage prepared in a similar manner to that of coleslaw and kept at 5°C for 25 days, and that the numbers had decreased only marginally after 64 days of storage. In contrast, the work also showed that heat treated coleslaw cabbage did not support the growth *L. monocytogenes* at 5°C, and the population steadily declined (Beuchat *et al.*, 1986). Work done by Dreux and co-workers (2007) showed that parsley that was surface-inoculated with *L. monocytogenes* showed around 4 log decreases within two days and concluded that contamination of aerial surfaces of the plant during irrigation would not result in produce that is contaminated. However, these workers took a value of  $10^3$  cfu.L<sup>-1</sup> *L. monocytogenes* in sewage water for irrigation as the worst case scenario (Dreux *et al.*, 2007).

Damaged tissue adds an additional contamination risk factor, as colonisation frequently occurs in these areas. *E. coli* O157:H7 has been isolated from bruised apple tissue, with growth occurring between two and six days after inoculation (Dingman, 2000). In addition, there are many instances in literature where cohabitation of organisms in damaged tissue has been reported to have a positive effect on pathogen growth. When *Salmonella typhimurium* was inoculated with a soft rot bacterium into potatoes, carrots and peppers, a ten-fold increase in counts were observed after 24 h when compared to *Salmonella* inoculation alone (Beuchat, 2002). The presence of *Pseudomonas viridiflava* lead to a three times higher increase in *Salmonella* counts when both organisms were inoculated into vegetable tissue (Beuchat, 2002). The co-infection of damaged plant tissue has also been reported to aid in the proliferation of pathogens, with the concentration

of *E. coli* O157:H7 increasing by 3 log<sub>10</sub> during co-infection of apple tissue with *Glomerella cingulata* (Riordan *et al.*, 2000).

Pathogen biofilms can occur on the surfaces of produce through the secretion of exopolysaccharides which, as stated previously, acts as a growth enhancing capsule for bacteria, yeasts and moulds. It appears to serve as a protective barrier that dramatically reduces the efficacy of sanitisers (Beuchat, 2002; Ryu & Beuchat, 2004). In a study by Beuchat (2002), *Salmonella* and *E. coli* O157:H7 were found to be protected by a biofilm to the extent where reduction with antimicrobial compounds was extremely difficult. Similarly, *L. monocytogenes* was unhindered by sanitising with 500 ppm free chlorine, when encapsulated in a multispecies biofilm with *Pseudomonas fragi* and *Staphylococcus xylosus* (Beuchat, 2002). It has also been shown that *L. monocytogenes* is able to grow and develop resistance to sodium hypochlorite when occurring in a multispecies biofilm (Norwood & Gilmour, 2000).

Furthermore, pathogens may become protected from sanitisers if they are internalised by the fruit. Burnett & Beuchat (2002) confirmed this, reporting that only a ten- to hundred-fold additional decrease in microbial numbers is attained with sanitisers as opposed to washing with water, which points to organisms being situated within the plant tissue. Therefore, when internalisation occurs, pathogens are not effectively removed and, as stated by Aruscavage *et al.* (2006), “*preharvest contamination control is potentially more critical than the best postharvest washing strategies*”. Internalisation may be avoided or reduced through temperature control, since this process can occur if the fruit is warmer than its inoculum thereby creating a negative temperature gradient (Kenney & Beuchat, 2002). Zhuang *et al.* (1995) also found that *Salmonella* Montevideo was more readily internalised in the cores of tomatoes at 25°C than 10°C.

As Beuchat (1996) states so eloquently: “...if consumers are to continue to benefit from the essential contribution raw and minimally processed vegetables make to their nutritional well-being, they will also very likely continue to consume vegetables containing low numbers of *L. monocytogenes*”, a statement which can be extended to a host of pathogens in South African irrigation water due to the current state of the country’s rivers.

## 2.4. INDICATORS OF FAECAL CONTAMINATION

The determination of microbiological quality could theoretically be done by testing for the presence of all the waterborne pathogens. However, this is not practically feasible in the routine laboratory. It is far more pragmatic to test for a single organism that can sensitively and specifically indicate the extent of faecal contamination or the efficacy of water treatment (WHO, 1993). These organisms are called indicator organisms, and verification of microbiological water quality usually includes testing for *E. coli* as an indicator of faecal contamination (WHO, 2011).



### 2.4.1. Suitability of indicators

The so-named indicator organisms are organisms, such as *E. coli*, that are used for routine assessment of the presence of pathogens in water (Barnes, 2003) since testing for each pathogen would be practically and economically unsound. These organisms should conform to a list of criteria, namely that they should:

- be suitable for all types of water;
- be present in sewage and polluted water whenever pathogens are present (Wilkes *et al.*, 2009);
- be present in numbers that correlate with the extent of pollution;
- be present in numbers higher than those of the pathogens;
- not multiply in the aquatic environment;
- be able to survive in the aquatic environment for at least as long as the pathogens;
- be absent from unpolluted water;
- be practically and reliably detected;
- be inexpensively and rapidly detected (Wilkes *et al.*, 2009);
- have transport characteristics that mimic those of pathogens (Wilkes *et al.*, 2009);
- and, not be pathogenic and safe to work with in the laboratory (DWAF, 1996; Barnes, 2003).

Thus far, no single organism has been found that conforms to all of these criteria. The “total coliform” group was historically used as an indicator of faecal contamination after the observation by Von Fritsch in the late 1800’s that *Klebsiella pneumoniae* and *K. rhinoscleromatis* are typically found in human faeces (Ashbolt *et al.*, 2001). Throughout the years, the group has been expanded to include many genera and today comprises *Arsenophonus*, *Budvicia*, *Buttiauxella*, *Cedecea*, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Erwinia*, *Escherichia*, *Ewingella*, *Hafnia*, *Klebsiella*, *Kluyvera*, *Leclercia*, *Leminorella*, *Moellerella*, *Morganella*, *Obesumbacterium*, *Pantoea*, *Photobacterium*, *Pragia*, *Proteus*, *Providencia*, *Rahnella*, *Salmonella*, *Serratia*, *Shigella*, *Tatumella*, *Trabulsiella*, *Xenorhabdus*, *Yersinia* and *Yokenella* (Leclerc *et al.*, 2001). As faecal indicator, however, the group has fallen from grace due to the presence of its members in environmental sources other than faeces (Ashbolt *et al.*, 2001; Leclerc *et al.*, 2001; Kämpfer *et al.*, 2008; Wutor *et al.*, 2009) and the ability of some coliforms to multiply in the environment (Kämpfer *et al.*, 2008). However, this group is still enumerated as a process indicator for the efficacy of chlorine disinfection (Ashbolt *et al.*, 2001). The group of organisms called the thermotolerant or “faecal” coliforms are commonly used as indicators of faecal pollution, and describe all bacteria which produce blue colonies on mFC agar at an incubation temperature of 44.5°C for 24 hours (DWAF, 1996). This, however, may prove to be problematic since not all faecal coliforms by name are faecal in origin. Bacteria such as *Citrobacter* (Kämpfer *et al.*, 2008), *Enterobacter* (Kämpfer *et al.*, 2008), *Erwinia* (Wutor *et al.*, 2009), *Klebsiella* (Kämpfer *et al.*, 2008), *Serratia* (Kämpfer *et al.*,

2008) and *Yersinia* (Wutor *et al.*, 2009) meet all the criteria as coliforms (aerobic and facultative anaerobic Gram negative rods which are unable to form spores and can ferment lactose to gas and acid at 35 °C after 48 hours (Rompré *et al.*, 2002)) but are not faecal in origin (Kämpfer *et al.*, 2008). Also, faecal coliforms in a water source may not indicate recent pollution but may be organisms that have been associated with sediments for a period of time without a loss of viability, as proven by LaLiberte and Grimes (1982). These two groups were replaced by *E. coli* which is found both ubiquitously (WHO, 1993) and, more importantly, highly specifically in mammalian faeces because of its exclusive inhabitancy of the intestines (Leclerc *et al.*, 2001). This organism does not multiply considerably in the environment (NHMRC & ARMCANZ, 1996) or receiving waters (Leclerc *et al.*, 2001) and thereby avoids the indication of faecal pollution too far downstream. These aspects show that *E. coli* is the best indicator of faecal pollution of a water source, a view which is confirmed by other workers (Edberg *et al.*, 2000; Murray *et al.*, 2007).

#### Characteristics of *E. coli*

*E. coli* is a non-pathogenic (Jagals *et al.*, 2006), thermotolerant coliform which belongs to the family *Enterobacteriaceae*. The organism can ferment mannitol and lactose to produce acid and gas, produces indole from tryptophane at 44°C (WHO, 1993), produces a positive methyl red test, cannot produce acetyl-methyl carbinol and does not use citrate as its only carbon source (Rompré *et al.*, 2002). It is also incapable of hydrolysing urea, and does not possess oxidase activity (WHO, 1993). As a coliform, *E. coli* produces the enzyme  $\beta$ -D-galactosidase (Chang *et al.*, 1989; Ashbolt *et al.*, 2001) which is encoded for by the *lacZ* gene and related to the fermentation of lactose (Ashbolt *et al.*, 2001) and the formation of gas, acid and aldehyde (Leclerc *et al.*, 2001). The majority of *E. coli* strains, however, also produce the more *E. coli*-specific (Maheux *et al.*, 2008) enzyme  $\beta$ -D-glucuronidase (Moberg, 1985; Chang *et al.*, 1989; Thompson *et al.*, 1990; De Boer & Beumer, 1999; Manafi, 2000; Ashbolt *et al.*, 2001) encoded for by the *uidA* gene (Feng *et al.*, 1991), a characteristic which is being used increasingly in the detection and enumeration of this organism since the concept was introduced by Edberg in 1988 (Ashbolt *et al.*, 2001). This enzyme functions by cleaving  $\beta$ -D-glucopyranosiduronic derivatives into their aglycon and D-glucuronic acid constituents (Alonso *et al.*, 1996). Due to its suitability and high specificity as faecal indicator (Omar *et al.*, 2009), a great deal of research time and money is devoted to improving the efficacy and rapidity of detection and enumeration which has led to a host of tests for *E. coli* in water (Rompré *et al.*, 2002; Jagals *et al.*, 2006).

One objection to the use of *E. coli* as an indicator is that the determination is based on specific detection which includes the number of harmless commensal *E. coli* and that these indicate the co-occurrence of pathogens. However, a high level of *E. coli* in water cannot unequivocally indicate that consumption will lead to disease with the South African Water Quality Guidelines utilising expressions such as “poses a significant risk of infection” (Jagals *et al.*, 2006). Commensal *E. coli* also possess the ability in some instances to naturalise and start multiplying in



their secondary environment in contrast to some pathogens (Wilkes *et al.*, 2009). Another criticism of *E. coli* as an indicator is a discrepancy in the similarity of the organism with pathogens in terms of cell size, quantity in faecal matter and environmental fitness (Wilkes *et al.*, 2009).

These objections to *E. coli* as the most popular indicator of faecal pollution in health-related water microbiology (Jagals *et al.*, 2006) have led to research into finding feasible alternatives as indicators of faecal contamination.

#### 2.4.2. Alternative faecal indicators

A host of other organisms have been named as possible indicator organisms for faecal pollution, such as enterococci (Harwood *et al.*, 2005; Murray *et al.*, 2007; Wutor *et al.*, 2009), *Bacteroides*, *Bifidobacterium*, endospore forming *Clostridium perfringens* (Harwood *et al.*, 2005) as well as phages like *Bacteroides fragilis* phage and F+ RNA coliphage (Savichtcheva & Okabe, 2006). In addition, the chemical compound coprostanol has also suggested as a possible alternative indicator (Leeming & Nichols, 1996; Savichtcheva & Okabe, 2006).

##### Enterococci

In comparison with *E. coli*, the enterococci group persists longer in the environment. Additionally, the detection of enterococci is, as with *E. coli*, easy and inexpensive which makes it a lucrative alternate indicator organism. However, persistence for a prolonged period of time could be detrimental to the assessment of faecal contamination as “old” organisms could point to faecal contamination that has occurred much higher upstream (Savichtcheva & Okabe, 2006). It is also well-known that enterococci are present in soil and dairy products in addition to mammalian intestines (Murray *et al.*, 2007). Plant matter can also be a source of enterococci such as *E. casseliflavus*, *E. faecalis* var. *liquefaciens*, *E. malodoratus* and *E. solitarius* (WHO, 1993). Reviews of the USEPA recreational water guidelines indicated that *E. coli* is superior to enterococci when predicting gastrointestinal illness from fresh water, but that the inverse is true for marine waters (Wilkes *et al.*, 2009). Experiments done by Sinton and his co-workers (2002) on sunlight inactivation of bacteria from waste stabilisation pond effluent showed that the inactivation rates ( $k_s$  – a function of global solar radiation) in river water was the highest for enterococci, followed by faecal coliforms and then *E. coli*. Effluent from winter months showed similar inactivation for faecal coliforms and enterococci, but that from summer months showed far higher inactivation rates for enterococci which are attributable to sunlight sensitivity acquired due to damage in the waste stabilisation pond (Sinton *et al.*, 2002).

### *Bacteroides*

*Bacteroides*, and particularly *Bacteroides* strain GB-124 (Ebdon *et al.*, 2007), has shown promise since it is extremely host-specific, but the anoxic requirement of the organism (Savichtcheva & Okabe, 2006) for traditional culture methods have made the analysis expensive and impractical. In addition, higher temperatures in summer months lead to an increase in degradation and predator activity and a subsequent marked decrease in *Bacteroides* numbers when employing PCR (Savichtcheva & Okabe, 2006). Also, the entire *Bacteroides* genus is not limited to human faecal matter (Ebdon *et al.*, 2007).

### *Bifidobacterium* spp.

Bifidobacteria have been considered as an alternative indicator because of their variability in animals and the fact that they can only be isolated from human and swine faeces (Savichtcheva & Okabe, 2006). However, their low recovery rate (60 - 70%) after more than three hours sample transit time, their rapid degradation (*ca.* 7 days) in warm water (23°C to 30°C) during summer months coupled with their inhibition by background microbes such as predators and Gram positive organisms (Savichtcheva & Okabe, 2006) have decreased their potential as faecal indicators.

### *Clostridium perfringens*

A current favourite, *Clostridium perfringens*, shows promise as an indicator as elevated temperatures and predation does not affect the numbers of the organism due to its ability to form endospores during adverse conditions (Savichtcheva & Okabe, 2006). A criticism of the organism for use as “indicator” is that it may, in fact, persist too well and indicate pollution far down from where it actually occurred (Savichtcheva & Okabe, 2006). This organism does not necessarily originate from a faecal source (WHO, 1993). Additionally, the concentration of *C. perfringens* in faeces varies considerably between animal species and humans (Savichtcheva & Okabe, 2006).

### *Bacteriodes fragilis* phage

The *Bacteriodes fragilis* bacteriophage has been suggested as it only multiplies in the intestine of humans, and the *B. fragilis* HSP 40 strain has not been detected in animal faeces (Savichtcheva & Okabe, 2006) whilst better surviving in water at 5°C and 25°C than the enteric viruses (Savichtcheva & Okabe, 2006). Additionally, phages have a much slower rate of decay when compared to faecal streptococci and faecal coliforms (Lucena *et al.*, 1996). However, it is very difficult to recover from lightly polluted water with a low level of faecal contamination (Savichtcheva & Okabe, 2006) which limits its practicality, outside of highly polluted water quality analyses.

### F+ RNA coliphage

For species specific assessment of faecal pollution in a water source, RNA coliphages have been suggested since the faeces from animals and humans contain differing serotypes (Savichtcheva & Okabe, 2006). For the purpose of detecting human faecal pollution, the F+ RNA coliphage has been considered due to its structural, dimensional and genetic similarities with enteric viruses (Savichtcheva & Okabe, 2006). However, these similarities have made them more appropriate for evaluating the viral contamination as opposed to general faecal contamination. This has been corroborated in work done by Ratto *et al.* (1989) in Peru, who found coliphages present in drinking water samples which were free from coliforms. These workers concluded that chlorination of these water sources was insufficient, and that the presence of coliphages indicated a likelihood that pathogenic viruses were also still present (Ratto *et al.*, 1989). In addition, their concentration and recovery is still a convoluted method at present (Savichtcheva & Okabe, 2006).

### Coprostanol

A chemical alternative to using micro-organisms for indicators of faecal pollution has also been proposed, namely coprostanol (Leeming & Nichols, 1996; Savichtcheva & Okabe, 2006). This organic compound of faecal origin is a sterol which is excreted by humans and animals and degrades microbiologically within 10 days at 20°C. This makes it specifically suitable for indicating fresh faecal pollution whilst the structurally similar sterols could point to the source of the contamination (Savichtcheva & Okabe, 2006). Workers reported that a level of 60 and 400 ng.L<sup>-1</sup> coprostanol corresponds to the primary (150 cfu.100 mL<sup>-1</sup>) and secondary (1 000 cfu.100 mL<sup>-1</sup>) contact limits for the traditional faecal indicators, namely faecal coliforms (Leeming & Nichols, 1996). However, in addition to a lack of proven correlation with pathogens, its association with particles and sewage due to its hydrophobicity could lead to the compound's inclusion in sediments where it could remain for up to 450 days without degradation, and therefore erroneously indicate contamination long after its occurrence (Savichtcheva & Okabe, 2006). Bartlett (1987) found the compound to be reduced by less than 15% after 30 days in sludge, and reported that coprostanol levels showed no reduction in sediments after 54 days. Moreover, there is insufficient epidemiological evidence to support the link of coprostanol and health risk (Leeming & Nichols, 1996).

### Comparison of alternative indicators with *E. coli*

Work done by Wilkes and co-workers (2009) on the relationship between indicator organisms and pathogens, including *Giardia* cysts and *Cryptosporidium* oocysts, showed that the relationship was indeed positive, albeit weakly so, with the exception of *L. monocytogenes* whose numbers were inversely related to indicator organisms. The results also indicated that of all the indicators tested

(*Clostridium perfringens*, enterococci, *E. coli*, total coliforms and faecal coliforms); *E. coli* was the best indicator of pathogen and parasite presence, with faecal coliforms a close second. Enterococci and total coliforms indicated pathogen and parasite presence to a much lesser extent (Wilkes *et al.*, 2009). Therefore, *E. coli* is by no stretch of the imagination the perfect indicator, as it is said not to persist very long outside of the intestine (Murray *et al.*, 2007). However, this organism is by far the best indicator of faecal pollution at present and performs the valuable task of inexpensively and rapidly assessing the faecal pollution of water as well as the efficacy of water treatment (Grabow *et al.*, 2004).

#### Inadequacies of known indicators in the prediction of viral presence

One field where all known bacterial indicators fall short is in the prediction of the presence of viruses in water. In untreated water, indicator organisms will outnumber viruses and thus predict an overestimation (Grabow *et al.*, 2004). However, viruses are considerably more resistant to disinfection (WHO, 2011) than traditional water quality indicator organisms which would result in underestimation of viral presence in disinfected water (Grabow *et al.*, 2004). Research by Grabow *et al.* (2004) suggested the use of the coxsackie B viruses as an “indicator” or model virus for water supplies as they occur in high numbers, which implies a reliable quantitative assessment. They are members of the group of enteric viruses and are detected easily and economically. Literature also cites F-RNA phages or coliphages as enteric virus models (Sinton *et al.*, 2002; Savichtcheva & Okabe, 2006). However, the same problem is encountered as during the enumeration of protozoan parasites, and for this purpose the WHO has recommended a system of water quality control relying on Hazard Analysis Critical Control Point (HACCP) instead of the traditional end-point assessment (Grabow *et al.*, 2004). This approach may be very effective in the assessment of treated water, but remains unfeasible in a system such as a river and therefore water quality assessment in this scenario continues to rely on the bacterial indicator *E. coli*.

## **2.5. DETECTION AND ENUMERATION OF COLIFORMS AND *E. COLI* AS INDICATORS OF QUALITY: MULTIPLE TUBE FERMENTATION**

Several “traditional” methods have been internationally approved for the detection and enumeration of coliforms and *E. coli* by regulatory bodies such as the USEPA and the French Standardization Association (AFNOR). Most of these methods are based on lactose fermentation and include the International Organization for Standardization (ISO) 9308-3 multi-well plate technique (Niemelä *et al.*, 2009), membrane filtration technique, the presence or absence test and the multiple tube fermentation (MTF) technique (Rompré *et al.*, 2002). Of these methods, MTF, which can enumerate faecal coliforms or *E. coli* with the assistance of most probable number

(MPN) tables (Edberg & Edberg, 1988; Christensen *et al.*, 2002) and statistical estimations, is by far the most accurate. It is the accuracy of the multiple tube fermentation method and the ability to statistically semi-quantify that has made it the method of choice even though it is expensive, labour intensive and less rapid, yielding results in three to four days (Maheux *et al.*, 2008).

### 2.5.1. MTF protocol

As shown in Table 2.6, the first step is inoculating a series of sample dilutions into lauryl sulphate tryptose broth at 35°C for 48 h (to select for organisms that grow at mammalian body temperature) as presumptive test for coliforms, with a positive test being lactose fermentation and gas formation.

Inocula from positive LST tubes are transferred to brilliant green lactose bile (BGLB) broth and incubated for 35°C at 48 h. The reason for incubation for 48 h is to promote the growth of slow-growing indicator bacteria from environmental samples (McFeters *et al.*, 1997). In BGLB, the brilliant green and bile inhibits the growth of *Aeromonas*, *Klebsiella*, *Shigella* and lactose-degrading clostridia while sodium lauryl sulphate acts as anionic surfactant. This test is the confirmation for coliforms, with a positive test being lactose fermentation and gas production. Although non-faecal coliforms can grow in this medium, they mostly do not produce gas (Anon., 2007).

Positive tubes are transferred to *Escherichia coli* (EC) broth which confirms coliforms as faecal coliforms or, more specifically, *E. coli* (Wang & Fiessel, 2008). Tubes are incubated at 44.5°C for 24 h. This temperature selects for thermotolerant coliforms and excludes *Klebsiella oxytoca*, which has the ability to yield false positive *E. coli* counts with indole confirmation (Pitkänen *et al.*, 2007). Lactose in the broth can be fermented by lactose-positive bacteria and bile salts inhibit the growth of Gram-positive bacteria that are not adapted to the intestinal environment. The 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG) supplement is added to select for organisms that possess the enzyme  $\beta$ -D-glucuronidase, and consequently hydrolyses MUG, a coumarin derivative (De Boer & Beumer, 1999), by cleaving the glycosidic bond between hydroxycoumarin 4-methylumbelliferone and glucuronic acid (Fior *et al.*, 2009). A fluorescent compound methylumbelliferone (MUF) (Lebaron *et al.*, 2005) is produced, which can be observed as a blue fluorescence under long-wave UV light (Covert *et al.*, 1989; Manafi *et al.*, 1991;) at 366 nm (Anon., 2007). MUG is favoured above other fluorinated umbelliferones which could potentially be toxic and due to its relative low cost as it is generally used for the detection of  $\beta$ -glucuronidase (Fior *et al.*, 2009). The expression of this enzyme is coded for by the *uidA* gene (Rompré *et al.*, 2002), specific in *E. coli* (Maheux *et al.*, 2008), and is regarded as a final hurdle in the detection of *E. coli* in EC broth. A MUG-containing medium such as EC broth should have a slightly elevated pH (Manafi *et al.*, 2000), since the pH optimum of *E. coli*  $\beta$ -D-glucuronidase is ca. 7.2 (Fricker *et al.*, 2010; Fang *et al.*, 1995) and MUF is very fluorogenic at pH values as high as 9.5 to 10.5 (Fang *et al.*, 1995). If the pH of the medium is not suitably alkaline, an alkali should be added before observing fluorescence (Manafi, 2000). EC tubes with gas production and fluorescence under UV light are a positive test for *E. coli*. These tubes are streaked out on Levine eosin methylene blue

(L-EMB) agar for final confirmation (Anon., 2007) after 24 h at 35°C. Dyes in this medium inhibit the growth of Gram-positive organisms (Anon., 2007). According to the manufacturer, *E. coli* colonies on L-EMB agar are dark with prominent metallic sheen (Anon., 2007).

**Table 2.6** Major steps and hurdles in the MTF method (adapted from Oxoid, 2001a; Oxoid, 2001b; Oxoid, 2001c; Oxoid, 2001d and Oxoid, 2001e; Christensen *et al.*, 2002; Anon., 2007)

Steps in the MTF method	Hurdles for selection	Select for	Positive reaction
Lauryl sulphate tryptose (LST) broth at 35°C for 48 h	<ul style="list-style-type: none"> <li>Fermentation of lactose with gas production</li> <li>Sodium lauryl sulphate surfactant acts as inhibitory agent for aerobic sporing bacteria and selects for the coliform group</li> <li>Intestinal temperature of 35°C</li> </ul>	Presumptive coliforms	Gas production in inverted Durham tube, turbidity
Brilliant green lactose bile (BGLB) broth at 35°C for 48 h	<ul style="list-style-type: none"> <li>Fermentation of lactose with gas production</li> <li>Bile excludes Gram positives</li> <li>Brilliant green excludes Gram-positives and anaerobic lactose reducers such as clostridia</li> <li>Intestinal temperature of 35°C</li> </ul>	Confirmed coliforms	Gas production in inverted Durham tube, turbidity
<i>Escherichia coli</i> (EC) broth with MUG at 44.5°C for 24 h	<ul style="list-style-type: none"> <li>Fermentation of lactose with gas production</li> <li>Bile to exclude spore formers, enterococci and Gram-positives not suited to the intestine (Anon., 2002)</li> <li>Thermotolerant coliforms selected with 44.5°C</li> <li>MUG select for anaerogenic strains of <i>E. coli</i> with <math>\beta</math>-D-glucuronidase</li> </ul>	<i>E. coli</i>	Gas production in inverted Durham tube, turbidity, fluorescence under long-wave UV light (366 nm)
Levine-eosin methylene blue (L-EMB) agar at 35°C for 24 h	<ul style="list-style-type: none"> <li><i>E. coli</i> shown as isolated colonies with a metallic green sheen imparted by the yellowish eosin and methylene blue dyes under reflected light, due to the acidic conditions created by lactose fermentation (Anon., 2004)</li> <li>Yellowish eosin and methylene blue dyes inhibit some strains of <i>Salmonella</i> and <i>Shigella</i></li> <li>Intestinal temperature of 35°C</li> </ul>	Confirms <i>E. coli</i>	Colonies with a metallic green sheen in reflected light and dark centres in transmitted light

### 2.5.2. Problems associated with MTF

Despite several selective hurdles in the MTF method, some concerns have been expressed regarding the accuracy of the method for detection and enumeration of coliforms and *E. coli*. These problems are attributable to limitations in both the detection criteria and the bacteria which are tested for, but can be broadly divided into problems associated with (1) detection by lactose fermentation; used in all four steps; and (2) detection by MUG hydrolysis; used in the EC broth step.

**Note:** during the discussion of this section, the terms “aerogenic” and “anaerogenic” have been used in the description of MTF target organisms. In this context, this does not refer to the oxygen requirements of the organism, but the ability or inability to produce gas from lactose media.

#### Problems with coliform and *E. coli* detection by lactose fermentation

One of the most important factors contributing to under-estimation with MTF is the presence of anaerogenic strains of coliforms in the water sample which is being analysed. These anaerogenic organisms fail to produce gas from lactose (Edberg *et al.*, 1988), which is incorporated in the media, and consequently remain undetected in accordance with the MTF protocol. LeChevallier *et al.* (1983) reported that only 58.9% of presumptive coliforms from contaminated surface water and drinking water samples were verified by gas production in lauryl tryptose broth (LTB), in comparison with the 84.6% verified by enzymatic detection of  $\beta$ -D-galactosidase and cytochrome oxidase. Apart from an intrinsic inability to produce gas from lactose, some initially aerogenic strains may not produce gas from lactose due to interference from autochthonous bacteria present in the sample. Gram negative bacteria outside of the coliform grouping (Edberg & Edberg, 1988) and heterotrophic bacteria at concentrations exceeding 500 cfu.mL<sup>-1</sup> (Evans *et al.*, 1981a) have been reported to have a detrimental impact on the sensitivity of the MTF method. Since *E. coli* is considered the faecal indicator of choice for water microbiology (Edberg *et al.*, 2000; Murray *et al.*, 2007), the occurrence of anaerogenic *E. coli* is of particular interest and has been reported in the literature (Meadows *et al.*, 1980; Evans *et al.*, 1981b; Leclerc *et al.*, 2001). Fricker *et al.* (1997) found that 10.0% of *E. coli* strains isolated from the Thames could not ferment lactose, a result which is similar to the approximation given by Szabo & Todd (1997) who estimate that 10.0% of all *E. coli* strains are either late lactose fermenters or completely anaerogenic. These organisms will remain undetected during a MTF analysis and consequently be excluded from the enumeration (Szabo & Todd, 1997). In turn, this under-estimation of the number of *E. coli* present in a sample will give rise to an under-appreciation of the faecal contamination of the sample.

While the selective hurdles indicated in Table 2.6 are specifically incorporated to exclude or inhibit false positive gas production by non-target organisms, an over-estimation may occur when such organisms synergistically produce a positive reaction. A high number of false positive presumptive coliforms have been observed in LST, which is attributable to the synergistic positive reactions produced by members of the enterococci and *Proteus* species (Schiff *et al.*, 1970). While further validation, as recommended by Edberg & Edberg (1988), will reduce the enumeration of false positives; an additional step will further increase the time until results are available and corrective action can be taken.



Problems with *E. coli* detection by MUG hydrolysis

Many literature sources indicate that the use of MUG hydrolysis by  $\beta$ -D-glucuronidase for the detection of *E. coli* has serious limitations. According to Balebona *et al.* (1990), *Klebsiella pneumoniae* is also able to fluoresce in the presence of MUG. This phenomenon is confirmed by Jagals and his co-workers (2006), who indicated that organisms from the genus *Salmonella*, *Shigella* and *Yersinia* possess the ability to hydrolyse MUG to fluorescent MUF, which could lead to overestimation during enumeration of *E. coli*. Between 17 and 29% of *Salmonella* species, half of all the *Shigella* species and several species of the *Yersinia* genus possess  $\beta$ -D-glucuronidase activity (Alonso *et al.*, 1996). The reports of these workers show that the production of  $\beta$ -D-glucuronidase, although it is reasonably highly associated with *E. coli*, is not a trait that belongs exclusively to this organism. Table 2.7 shows a summary of all genera which contains strains with the ability to produce  $\beta$ -D-glucuronidase. References in this table refer to identification of problematic genera, while references with a superscript indicate the identification of a particular species within the genus.

**Table 2.7** Organisms with the ability to produce  $\beta$ -D-glucuronidase

Genus	Species	Reference
<i>Aerococcus</i>	Not specified	Fricker <i>et al.</i> , 2010
<i>Aeromonas</i>	Not specified	Geissler <i>et al.</i> , 2000; Pisciotta <i>et al.</i> , 2002
<i>Bacillus</i>	Not specified	Fricker <i>et al.</i> , 2010
<i>Bacteriodes</i>	Not specified	Dahlén & Linde, 1973; Fricker <i>et al.</i> , 2010
<i>Citrobacter</i>	<i>C. freundii</i> <sup>a</sup>	<sup>a</sup> Chang <i>et al.</i> , 1989; <sup>a</sup> Geissler <i>et al.</i> , 2000; <sup>a</sup> Manafi, 2000; Fricker <i>et al.</i> , 2010
<i>Clostridium</i>	Not specified	Manafi <i>et al.</i> , 1991; Fricker <i>et al.</i> , 2010
<i>Corynebacterium</i>	Not specified	Dahlén & Linde, 1973
<i>Enterobacter</i>	<i>E. agglomerans</i> <sup>b</sup>	<sup>b</sup> Geissler <i>et al.</i> , 2000
<i>Flavobacterium</i>	Not specified	Covert <i>et al.</i> , 1989; Manafi <i>et al.</i> , 1991; Pisciotta <i>et al.</i> , 2002
<i>Hafnia</i>	Not specified	Fricker <i>et al.</i> , 2010
<i>Klebsiella</i>	<i>K. pneumoniae</i> <sup>c</sup>	<sup>c</sup> Chang <i>et al.</i> , 1989; <sup>c</sup> Balebona <i>et al.</i> , 1990
	<i>K. oxytoca</i> <sup>d</sup>	<sup>d</sup> Manafi, 2000
	<i>K. ozaenae</i> <sup>e</sup>	<sup>e</sup> Chang <i>et al.</i> , 1989
<i>Peptostreptococcus</i>	Not specified	Fricker <i>et al.</i> , 2010
<i>Photobacterium</i>	<i>P. damsela</i> <sup>f</sup>	<sup>f</sup> Pisciotta <i>et al.</i> , 2002
<i>Providencia</i>	Not specified	Pisciotta <i>et al.</i> , 2002
<i>Pseudomonas</i>	Not specified	Pisciotta <i>et al.</i> , 2002

**Table 2.7 continued**



Table 2.7 continued

Genus	Species	Reference
<i>Salmonella</i>	Not specified	Hansen & Yourassowsky, 1984; Feng <i>et al.</i> , 1991; Manafi <i>et al.</i> , 1991; Alonso <i>et al.</i> , 1996; Manafi, 2000; Pisciotto <i>et al.</i> , 2002; Jagals <i>et al.</i> , 2006; Fricker <i>et al.</i> , 2010
<i>Serratia</i>	<i>S. fonticola</i> <sup>g</sup>	<sup>g</sup> Manafi, 2000
<i>Shigella</i>	<i>S. sonnei</i> <sup>h</sup>	<sup>h</sup> Hansen & Yourassowsky, 1984; Feng <i>et al.</i> , 1991; Manafi <i>et al.</i> , 1991; Alonso <i>et al.</i> , 1996; Manafi, 2000; Pisciotto <i>et al.</i> , 2002; Jagals <i>et al.</i> , 2006; Fricker <i>et al.</i> , 2010
<i>Staphylococcus</i>	Not specified	Manafi <i>et al.</i> , 1991; Fricker <i>et al.</i> , 2010
<i>Vibrio</i>	Not specified	Geissler <i>et al.</i> , 2000; Pisciotto <i>et al.</i> , 2002
<i>Yersinia</i>	<i>Y. intermedia</i> <sup>i</sup>	Manafi <i>et al.</i> , 1991; Alonso <i>et al.</i> , 1996; <sup>i</sup> Manafi, 2000; Jagals <i>et al.</i> , 2006; Fricker <i>et al.</i> , 2010

Apart from the problematic genera and species indicated in Table 2.7, detection of *E. coli* through the assay of the presence  $\beta$ -D-glucuronidase can be complicated by a variety of other factors. This enzyme is also a ubiquitous feature in the lysozymes of mammals (Fricker *et al.*, 2010), which can lead to its detection in the absence of *E. coli*. Furthermore, certain strains in the genus *Pseudomonas* can produce a greenish fluorescence, similar to that of MUF, under UV light (Geissler *et al.*, 2000; Manafi *et al.*, 1991). The presence of such non-target sources of  $\beta$ -D-glucuronidase in the sample will result in an over-estimation of *E. coli* numbers, thereby negatively impacting on the accuracy of the MTF enumeration.

In addition to the problematic detection of non-target  $\beta$ -D-glucuronidase, some strains of *E. coli* cannot be detected through a fluorogenic assay using MUG due to a lack of functional  $\beta$ -D-glucuronidase. According to Pisciotto *et al.* (2002), up to 20% of environmental *E. coli* strains may not be able to produce the enzyme, while Chang *et al.* (1989) reported that 34% of human faecal *E. coli* is  $\beta$ -D-glucuronidase negative. Other workers found that in *E. coli* from clinical sources the percentage of  $\beta$ -D-glucuronidase positive strains could be as high as 94% (Hansen & Yourassowsky, 1984). The latter is confirmed in literature by others, reporting positive  $\beta$ -D-glucuronidase *E. coli* proportions of 94% (Feng *et al.*, 1991), 94-96% (Manafi, 2000; De Boer & Beumer, 1999; Manafi *et al.*, 1991), 96% (Thompson *et al.*, 1990) and 97% (Moberg, 1985). It is also a well-known fact that the enterohaemorrhagic *E. coli* O157:H7 cannot produce  $\beta$ -D-glucuronidase (Omar *et al.*, 2010; Maheux *et al.*, 2008; Manafi, 2000; De Boer & Beumer, 1999; Feng *et al.*, 1991; Thompson *et al.*, 1990) due to the absence of *uidA* in its genome (Rompré *et al.*, 2002). Other strains of *E. coli* possess *uidA*, but do not express the gene to produce  $\beta$ -D-glucuronidase (Rompré *et al.*, 2002). Moreover, workers found that the entire enterohaemorrhagic *E. coli* group (Jagals *et al.*, 2006; Feng *et al.*, 1991), as well as sublethally injured *E. coli* (Jagals *et*

*al.*, 2006), cannot produce the enzyme; and Hansen & Yourassowsky (1984) reported that the enzyme is lacking in many strains belonging to the O1:K1 serotype. In a study determining the *uidA* sequences of  $\beta$ -D-glucuronidase negative *E. coli*, Feng *et al.* (1991) found that the majority, if not all strains, of *E. coli* possess the *uidA* gene. This was confirmed by Alonso *et al.* (1996) and Maheux *et al.* (2008) who reported that only 97% of *E. coli* isolates possess the *uidA* gene and therefore  $\beta$ -D-glucuronidase activity.

Feng *et al.* (1991) concluded that the lack of production of the enzyme could be the consequence of physical or environmental conditions, lactose-induced catabolite repression, or mutations in the genome which could result in non-functional  $\beta$ -D-glucuronidase. These problematic factors could lead to an underestimation of the number of *E. coli* organisms in water. Wutor *et al.* (2009) also reported unreliable results in their study of  $\beta$ -D-glucuronidase reduction of MUG, proposing that pollutants could interfere with the enzyme substrate. These factors may lead to the non-detection of some *E. coli* strains, which will lead to an under-estimation of the number of *E. coli* present in the sample. The under-estimation is detrimental to the accuracy of the MTF method and will lead to an under-estimation of the level of faecal contamination present in the sample.

#### General factors impacting negatively on the enumeration of faecal indicator bacteria (FIB)

Apart from complications arising from imperfect detection methods and indicator organisms, any risk assessment by water quality analyses is confounded by the multiple sources of faecal microorganisms (Wilkes *et al.*, 2009). Also, it is postulated that microbes can survive adverse conditions by entering a state known as viable but non culturable (Schultz-Fademrecht *et al.*, 2008), which would affect the accuracy of enumeration. This makes interpretations of results, pathogen predictions, difficult (Wilkes *et al.*, 2009) and final conclusions must be done extremely carefully. While these factors are problematic for all methods which enumerate FIB, their impact on the accuracy of MTF is of particular importance. Both local and international guidelines for assessing faecal contamination in water and food are based on *E. coli* numbers, and exceeding of such guidelines are legally prosecuted based on enumerations using MTF. The negative factors discussed under section 2.5.2 have a detrimental effect on enumeration with this method, which can prove problematic to national and international governing bodies employing the MTF method, since all legal arguments with MTF data will be under suspicion.

## **2.6. ALTERNATIVE METHODS OF DETECTION AND ENUMERATION FOR COLIFORMS AND *E. COLI* FROM WATER**

There are several alternative methods available for the detection of coliforms and *E. coli*, each with advantages and disadvantages. While some of these alternative methods are based on lactose

fermentation, a variety of methods relying on the chromogenic or fluorogenic (Manafi, 2000) detection of target bacterial enzymes or metabolites (Ashbolt *et al.*, 2001) are also becoming, or are already, commercially available. Additionally, there are also a host of emerging chemical, bioluminescent, immunological and molecular techniques for the detection of *E. coli*.

### 2.6.1. Alternative methods based on lactose media

In some countries, the membrane filter technique is preferred over the MTF method as it produces more rapid results and is associated with lower costs. The method involves filtering 100 mL of sample through a 0.45 µm filter and incubating it on any of a number of selective media. In Europe and North America, Tergitol-2,3,5-triphenyltetrazolium chloride (TTC) and m-Endo agar with lactose are most typically used. On the m-Endo medium, coliforms form red colonies with a metallic sheen at an incubation temperature of 35°C for 24 h. The m-Endo medium has limitations with atypical dark red, mucoid colonies with a metallic sheen growing on the medium; while some typical colonies may be produced by non-coliform bacteria and some atypical colonies being produced by coliforms (Rompré *et al.*, 2002). On Tergitol-TTC, the 2,3,5-triphenyltetrazolium chloride turns colonies of coliforms dark red (McLain *et al.*, 2008) and organisms produce yellow-orange colonies when incubated at 37°C for 24 h and 44°C for 48 h for total coliforms and thermotolerant coliforms, respectively. In Britain and South Africa, membrane filter methods are used in conjunction with Teepol or MacConkey agar, but research (Grabow & du Preez, 1979) showed that m-Endo agar produces higher counts than MacConkey or Teepol agar (Rompré *et al.*, 2002). For the exclusive enumeration of thermotolerant ("faecal") coliforms membrane faecal coliform (mFC) agar is recommended, since it is an enriched lactose medium with rosolic acid salt reagent to inhibit the growth of non-thermotolerant coliforms at an incubation temperature of 44.5°C for 24 h. However, Maheux *et al.* (2008) stated that m-Endo, mFC and Tergitol-TTC are lacking in specificity and further confirmatory measures need to be taken whilst a high degree of background microbes hinders coliform recovery.

#### Methods for the recovery of sublethally injured coliforms

For the recovery of stressed or sublethally injured coliforms, mFC agar can be used with the addition of catalase or sodium pyruvate to the medium, since injured bacteria are unable to degrade hydrogen peroxide due to a loss of catalase enzymatic activity (Rompré *et al.*, 2002). Since injured cells are more sensitive to surface-active reagents such as Tergitol 7, which is used for the inhibition of Gram-positive bacteria (Pitkänen *et al.*, 2007), another medium that is specifically effective in recovering chlorine stressed coliforms is m-T7 agar. This effect is, however, only when coliforms are affected by chlorine and no significant improvement over m-Endo LES or m-Endo was reported in organisms injured by monochloraminated water or ozone (Rompré *et al.*, 2002). McFeters and co-workers (1997) reported that the recovery of chlorine stressed coliforms with *E.coli* Colisure medium was higher when compared to MTF using lauryl

sulphate tryptose and subsequent incubation in brilliant green lactose bile or EC medium with added MUG.

### 2.6.2. Alternative methods based on chromogenic and fluorogenic enzyme detection

In the past few years, the use of enzymatic methods for the detection and enumeration of coliforms and *E. coli* has become very popular (Maheux *et al.*, 2008) and has gained acceptance for the assessment of water quality (McFeters *et al.*, 1997). These positive perceptions are mainly attributable to the greater exclusion of background heterotrophs by these methods, as well as the elimination of a subsequent confirmatory step (Maheux *et al.*, 2008).

The chromogenic and fluorogenic methods for the detection of coliforms and *E. coli* primarily rely on the action of two enzymes. The first enzyme, produced by most coliforms, is  $\beta$ -D-galactosidase (Ashbolt *et al.*, 2001; Chang *et al.*, 1989). This enzyme is encoded for by the *lacZ* gene and is involved in the fermentation of lactose (Ashbolt *et al.*, 2001) to yield acid, aldehyde and CO<sub>2</sub> gas (Leclerc *et al.*, 2001). It is also this production of CO<sub>2</sub> gas which is used as a positive reaction in certain steps of the MTF method. The second enzyme,  $\beta$ -D-glucuronidase, is more specific to *E. coli* although literature sources report that strains within a variety of other genera may also possess this enzyme (see Table 2.7 and section 2.5.2). This enzyme, as discussed under section 2.4.1, is encoded for by the *uidA* gene (Feng *et al.*, 1991) and is responsible for the hydrolysis of  $\beta$ -D-glucopyranosiduronic derivatives into their component aglycon and D-glucuronic acid (Alonso *et al.*, 1996).

Chromogenic methods for the detection of coliforms and *E. coli*

#### *m-Colibblue 24 broth*

A chromogenic medium that has been recommended for the detection of coliforms and *E. coli* is the m-Colibblue 24 broth (containing TTC and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide). In this medium, coliforms are typified by red colonies while *E. coli* produces blue colonies due to the utilisation of TTC (Wang & Fiessel, 2008). McLain *et al.* (2008) found that this broth yielded a high number of false positives when testing water from a wetland receiving tertiary treated municipal effluent. The false positives reported were around 35.0% in summer and 75.0% in winter.

#### *Chromocult coliform agar (CCA) and differential coliform agar (DCA)*

Chromocult coliform agar (CCA) (containing  $\beta$ -D-galactosidase, X-glucuronide and cefsulodin) can also be used (Maheux *et al.*, 2008) in the chromogenic detection of coliforms and *E. coli*. On this medium coliforms form pink to red colonies due to the presence of  $\beta$ -D-galactosidase, and *E. coli* forms dark blue to violet colonies due to the presence of both  $\beta$ -D-galactosidase and  $\beta$ -D-glucuronidase (Wang & Fiessel, 2008). Another medium relying on coliform detection by galactosidase activity is differential coliform agar (DCA; containing 5-bromo-4-chloro-3-indolyl- $\beta$ -D-

galactosidase and cefsulodin). DCA displays coliforms as light purple to white colonies with a pink rim and *E. coli* as a blue-centred red colony (Wang & Fiessel, 2008). As with CCA, cefsulodin is added to DCA as an inhibitor of background bacteria. However, in comparison with m-Endo medium CCA still has a high degree of background interference and the presumptive *E. coli* colonies needs to be confirmed with Kovacs reagent (production of indole from tryptophane) (Wang & Fiessel, 2008) and cytochrome oxidase (Pitkänen *et al.*, 2007). DCA, however, shows more effective suppression of background growth compared to m-Endo agar, while its ability to enumerate coliforms and *E. coli* also compared favourably (Wang & Fiessel, 2008).

### *CHROMagar E. coli*

CHROMagar *E. coli* (containing X-glucuronide and methyl-glucuronide) shows *E. coli* colonies as blue colonies after incubation at 44.5°C for 24 h. Alonso *et al.* (1996) reported no significant difference to m-FC agar in the ability of the medium to detect for *E. coli*. The same authors observed a reduced number of false negative *E. coli* colonies on CHROMagar *E. coli* with incubation at 37°C rather than 44.5°C (Alonso *et al.*, 1996). There are several limitations associated with the use of chromogenic media for the detection and enumeration of coliforms and *E. coli*. These include the interference of substances such as humic acids or a dominating naturally occurring organism in the water (McLain *et al.*, 2008). Also, the age of the reagents used play an important role, as old reagents are said to yield a higher number of false positives (McLain *et al.*, 2008). Furthermore, some water sources such as reclaimed water may contain chemicals that could disrupt the proper functioning of chromogenic media, such as endocrine disruptors, nutrients, salts and pharmaceuticals (McLain *et al.*, 2008). Apart from these reasons, the mediums are also extremely expensive and completely unaffordable for routine assessments.

Chromogenic/fluorogenic method for the detection of coliforms and *E. coli*

### *Colilert-18*

The defined substrate technology method registered as Colilert has been commercially in existence for over twenty years (Covert *et al.*, 1989). In spite of this, it has only recently, after improved automation, gained popularity for the rapid enumeration of both coliforms and *E. coli*. Detection is based on the production of  $\beta$ -D-galactosidase and  $\beta$ -D-glucuronidase by coliforms and *E. coli*, respectively, through the hydrolysis of a chromogenic and a fluorogenic compound by the two enzymes. Ortho (*o*)-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) (Rompré *et al.*, 2002; Kämpfer *et al.*, 2008), an *o*-nitrophenyl ester (Manafi *et al.*, 1991), is hydrolysed by  $\beta$ -D-galactosidase to liberate yellow *o*-nitrophenyl (Covert *et al.*, 1989). For the detection of *E. coli*, MUG (Rompré *et al.*, 2002; Kämpfer *et al.*, 2008), a coumarin derivative (De Boer & Beumer, 1999), is hydrolysed by  $\beta$ -D-glucuronidase to yield 4-methylumbelliferone (MUF) which has blue

fluorescence under long-wave UV light (Manafi *et al.*, 1991; Covert *et al.*, 1989). The two compounds form the Colilert reagent, which is commercially available in a sealed, powdered form.

The method shows considerable advantages over MTF, since analysis is far more automated and results can be read 18-22 hours later (Fricker *et al.*, 2008b; Wohlsen *et al.*, 2008). Furthermore, anaerogenic coliforms (Fricker *et al.*, 1997) do not affect the results (Wohlsen *et al.*, 2008) as is the case with MTF, which relies on the production of gas via lactose fermentation. Colilert also holds an advantage over traditional methods since the number of *E. coli* strains that cannot grow at 44°C or ferment lactose is greater than the number of *E. coli* strains which do not produce  $\beta$ -D-glucuronidase (Niemelä *et al.*, 2003; Wohlsen *et al.*, 2008). Fricker *et al.* (2008a) also reported that as many as 3.0% of *E. coli* cannot produce indole from tryptophane at 44°C, but that 99.5% of *E. coli* strains that were recovered from water could produce  $\beta$ -D-glucuronidase. *E. coli* O157:H7 (which is  $\beta$ -D-glucuronidase negative) has also been reported to grow very slowly, if at all, at 44°C (Fricker *et al.*, 1997). It has even been stated by some workers that the incubation of *E. coli* at 44°C, a common practice to enumerate thermotolerant coliforms, could cause significant reductions in the amount of *E. coli* detected and under-estimations of up to 30.0% (Fricker *et al.*, 2008a). In addition, the  $\beta$ -D-glucuronidase reaction is also less pronounced in *E. coli* which has been grown in media with a high amount of lactose (Fricker *et al.*, 2008b) which *E. coli* use preferentially over MUG (Niemelä *et al.*, 2003), and the Colilert reagent consists only of the two enzyme substrates thereby obligating the utilisation of MUG by *E. coli*.

Initially, however, the method was designed as a presence/absence analysis indicated by the presence or absence of yellow colour development or fluorescence. Due to its inability to enumerate, Colilert could not compete with MTF, irrespective of its benefits. This problem was solved with the introduction of the Quanti-Tray, a tray with 49 large and 48 small wells (1.6 mL and 120  $\mu$ L, respectively) (Pisciotta *et al.*, 2002) which can enumerate up to 2 419 total coliforms or *E. coli* per 100 mL (Wohlsen *et al.*, 2008) with the aid of a MPN table. The observation by some workers that chlorine-injured *E. coli* was not enumerated effectively by Colilert (Clark *et al.*, 1991) resulted in further improvements in the formulation and the introduction of the Colilert-18 reagent (Wohlsen *et al.*, 2008).

The development of the Quanti-Tray enabled Colilert to compete with enumeration methods for *E. coli*, such as MTF, and research was done internationally to compare Colilert with MTF (Eckner, 1998; Kämpfer *et al.*, 2008); membrane filtration on membrane lauryl sulphate broth (MLSB) (Fricker *et al.*, 1997), Tergitol-TTC agar (Niemelä *et al.*, 2003; Fricker *et al.*, 2008a), lactose TTC agar (Kämpfer *et al.*, 2008), mFC agar (Wohlsen *et al.*, 2008) and membrane lactose glucuronide agar (MLGA) (Fricker *et al.*, 2010). Comparisons were done using samples of laboratory-prepared bacterial culture solutions, drinking water, chlorinated water, groundwater, surface water and sewage effluent. All of these reports concluded that Colilert was a viable alternative to the method against which it was compared. They reported higher recovery rates for *E. coli* cultures against mFC agar ( $p \leq 0.0001$ ) (Wohlsen *et al.*, 2008) and MLGA (Fricker *et al.*,



2010); and similar sensitivity for *E. coli* and higher sensitivity for coliforms when compared against MTF (Eckner, 1998; Kämpfer *et al.*, 2008) and MLSB (Fricker *et al.*, 1997) for drinking water. Furthermore, a reduction in false positive results was observed compared to Tergitol-TTC in sewage effluent (Fricker *et al.*, 2008a); and higher recoveries of both coliforms and *E. coli*, when compared to Tergitol-TTC, for a variety of water samples originating from 13 European countries (Niemelä *et al.*, 2003) were also found. In 2009, Niemelä and co-workers compared Colilert-18 with the European Commission's recommended method for *E. coli*, the multi-well plate technique. This method is also based on the ability of *E. coli* to hydrolyse MUG and enumeration by MPN (Niemelä *et al.*, 2009). After analysis of fresh and seawater samples, verification of "positive" wells for both methods was done on TBX agar and verification rates of 97.8% for Colilert and 95.7% for multi-well plate were reported. Due to these favourable results, Colilert-18 has been accepted as an alternative to the multiple tube fermentation by the German Environmental Protection Agency (Kämpfer *et al.*, 2008), and has also subsequently been recommended as an alternative official method to the multi-well plate technique (Niemelä *et al.*, 2009).

However, work done in Finland showed that Colilert excluded all unwanted Gram-positive cocci, but that 30% of yellow but non-fluorescing wells still contained *E. coli* (Pitkänen *et al.*, 2007), and as a result undercounting was found. Other literature suggests an even higher number of ca. 81% of false negatives for *E. coli* when using Colilert (McFeters *et al.*, 1997). This is in agreement with literature indicating that some strains of *E. coli* do not possess  $\beta$ -D-glucuronidase, which creates the same concern for undercounts as with the traditional MTF method.

In addition, as with any microbiological method, Colilert-18 also has certain limitations and will perform according to its appropriateness for the characteristics of the sample that is being tested. For example, a study done by Pisciotta *et al.* (2002) on the enumeration of *E. coli* from marine waters in Florida found that when native marine bacteria with the ability to produce  $\beta$ -D-glucuronidase (*Photobacterium damsela*, *Providencia* sp. and *Vibrio alginolyticus*) and  $\beta$ -D-galactosidase (*Burkholderia cepacia* and *Vibrio cholerae*) occur in the same well, they could lead to serious overestimations, as high as 75.0% to 100.0%, of *E. coli*. This is reflected in the report by Eckner (1998), which stated that  $\beta$ -D-galactosidase and  $\beta$ -D-glucuronidase from algal sources or marine vibrios can interfere with the identification of coliforms. Geissler *et al.* (2000) made a similar observation: that marine vibrios can cause false positive reactions through their production of  $\beta$ -D-galactosidase and due to their competitive advantage as autochthonous bacteria in seawater. The study done by Pisciotta *et al.* (2002) also concluded that water salinity played an important role in the false positive results generated by these organisms. Highly diluted marine (1:20) water required a thousand-fold increase in bacterial concentration before producing the same number of false positives when compared to slightly diluted (1:10) seawater. Fricker *et al.* (2008b) also recommended that false positive reactions created by native organisms be remedied by incubation at 44 °C when analysing marine waters.

Another criticism of the Colilert MPN is its inability to distinguish between pathogenic and commensal strains of *E. coli* (Omar *et al.*, 2009). In 2009, Omar and co-workers used single multiplex PCR to determine the amount of pathogenic *E. coli* strains in *E. coli* positive Colilert wells. They reported that positive Colilert wells from sources such as springs, taps, rivers, raw sewage, stool and soil contained 17% enteroaggregative *E. coli*, 4% enteroinvasive *E. coli*, 21% enterotoxigenic *E. coli*, 17% enterohaemorrhagic *E. coli* and 21% enterohaemorrhagic and enteropathogenic *E. coli* (Omar *et al.*, 2009). While the detection of *E. coli* without further pathogenic characterisation can be problematic in fully understanding the risk posed by the faecal contamination (Ahmed *et al.*, 2007), this problem is not unique to Colilert-18 and can be extended to all of the methods previously discussed.

### 2.6.3. Alternative method based on chemical detection

The hydrogen sulphide (H<sub>2</sub>S) test has also received attention as a simple and rapid (Manja *et al.*, 1982) as well as reliable and inexpensive (Kromoredjo & Fujioka, 1991; Izadi *et al.*, 2010) alternative to assess the microbiological quality of drinking water. The test is based on the detection of H<sub>2</sub>S gas, anaerobically produced by proteolytic bacteria from sulphur-containing amino acids (Gabriel, 2005), with lead acetate impregnated test strips. The presence of these bacteria in water has been demonstrated to be associated with the presence of coliforms (Manja *et al.*, 1982) in a field study conducted in India. Despite the potential of this method as a rapid alternative method, most literature to date have focused on its applicability in drinking water and its performance in more polluted samples were unknown. However, recent work done by McMahan (2011) evaluated the potential of this test as an indicator of faecal contamination in water due to uncertainty pertaining to its sensitivity and specificity. This work explored a wider variety of water samples, including sewage water, and corroborated that the presence of H<sub>2</sub>S gas indicated the presence of faecal bacteria (McMahan, 2011).

### 2.6.4. Comparative studies with alternative methods

In research done by Pitkänen and co-workers in 2007, a number of coliform and *E. coli* enumeration techniques were compared with the membrane filtration ISO 9308-1 (2000) method on LTTC Tergitol 7 agar. They included LES Endo agar (the standard in Finland), Colilert-18 MPN method, Harlequin *E. coli*/coliform medium and chromogenic *E. coli*/coliform medium. The Harlequin and LES Endo mediums proved to have a very low amount of background interference when compared to the LTTC. It was also found that the chromogenic media overestimated the number of coliforms due to the presence of oxidase positive bacteria that do possess  $\beta$ -D-galactosidase activity.

Colilert, CCA, Readycult Coliforms 100 and MI agar (a membrane filtration agar) were also evaluated and compared by Maheux and co-workers (2008). They found Colilert to be the weakest method for testing  $\beta$ -D-glucuronidase (58% in comparison with yields ranging between 89.2 and



90.8%), and MI agar the worst for  $\beta$ -galactosidase. Their work also indicated that all of these methods are not appropriate for identification of presumptive *E. coli* strains due to the high incidence of false negatives (Maheux *et al.*, 2008).

Comparative studies done with the H<sub>2</sub>S test showed that this method had 71.0% correlation with standard MTF analyses for detecting *E. coli* in drinking water from Iran (Izadi *et al.*, 2010), but only 56.0% correlation with *E. coli* medium for the detection of faecal coliforms (Izadi *et al.*, 2010). Ratto *et al.* (1989) found the test to be equal to or more sensitive than MTF in the detection of coliforms and faecal coliforms in drinking water from Peru. Similarly, Manja *et al.* (1982) found a good agreement between the H<sub>2</sub>S test and MTF for the detection of coliforms in drinking water from India. In Indonesia, Kromoredjo and Fujioka (1991) reported that the H<sub>2</sub>S test and LTB with MUG (testing for *E. coli*) was positive for 47.8% and 45.7%, respectively, of tested drinking water samples. These workers also found an excellent agreement between H<sub>2</sub>S test and Colilert results (Kromoredjo & Fujioka, 1991). In addition, an in-depth study into the potential of the H<sub>2</sub>S test as an indicator of faecal contamination in water showed that the H<sub>2</sub>S test and standard *E. coli* detection methods had similar relationships with the incidence of diarrhoea (McMahan, 2011).

#### 2.6.5. Bioluminescent and immunological methods

Several methods based on bioluminescence and immunological reactions are available for the detection of coliforms and, especially, *E. coli*. Bioluminescent techniques such as flow cytometry (De Boer & Beumer, 1999), direct epifluorescent filter technique and adenosine-5'-phosphate bioluminescence (De Boer & Beumer, 1999) are increasingly used for detection, especially in food environments, as scientific and technological advances reduce the costs of these methods. Among the immunological methods are monoclonal and polyclonal antibody techniques, such as enzyme-linked immunosorbent assay (ELISA), (De Boer & Beumer, 1999; Ashbolt *et al.*, 2001) which can be used for detection through immune response. Other immune-based detection methods are immunomagnetic separation (IMS) (Ashbolt *et al.*, 2001) and *in-situ* hybridisation (ISH) (Rompré *et al.*, 2002), or fluorescence *in-situ* hybridisation (FISH) (Ashbolt *et al.*, 2001; Girones *et al.*, 2010).

Some of these methods show distinct advantages over traditional methods for the detection of *E. coli* from water and food, with IMS and FISH possessing the ability to detect viable cells even if they are traditionally unculturable (Ashbolt *et al.*, 2001). FISH, in particular, shows great promise for generating quantitative data, but requires more research (Barnes, 2003). However, limitations associated with these methods have also been identified: for example; ELISA techniques have pathogen detection limits between  $10^3$  and  $10^5$  cfu.mL<sup>-1</sup> (De Boer & Beumer, 1999) which severely impact on the applicability of the method. In addition, although many of these methods hold considerable advantages over traditional methods, they are typically unaffordable for routine analyses and few have the potential to become standardised laboratory methods for the detection of *E. coli*. They are, therefore, unsustainable in low-technology environments, such as rural municipalities.

### 2.6.6. Molecular methods

Molecular methods such as polymerase chain reaction (PCR) and molecular subtyping using techniques like restriction fragment length polymorphism (RFLP) are also being used for the detection of coliforms and *E. coli*. These methods are often considered more appropriate than traditional cultivation methods relying on phenotypic reactions. While the genotype of the organism is responsible for all observed phenotypic traits not all genetic information will necessarily be expressed, for example, the failure by some strains of *E. coli* to produce  $\beta$ -D-glucuronidase despite the presence of *uidA* in their genome (Rompré *et al.*, 2002). For these reasons, it has been stated that the presence of *uidA* is more reliable than determining  $\beta$ -D-glucuronidase in the detection of *E. coli* (Bej *et al.*, 1991; Heijnen & Medema, 2006). Historically, however, molecular methods for the detection of coliforms (including *E. coli*) concentrated on the detection of the *lacZ* gene, which encodes for the  $\beta$ -D-galactosidase enzyme (Almadidy *et al.*, 2002; Rompré *et al.*, 2002) associated with the fermentation of lactose. Bej *et al.* (1990) showed that the detection of *lacZ* was specific for coliforms, and the method was also demonstrated to produce results with no significant difference from those obtained by plating and defined substrate methods (Bej *et al.*, 1991). Despite these advantages the detection of *lacZ* has become relatively obsolete in risk assessments since the coliform group has been replaced by *E. coli* as a more specific indicator of faecal pollution.

For the purpose of detecting *E. coli* exclusively, the presence of the *tuf* gene has been suggested as an alternative to the presence of *uidA*. This gene is associated with the Tu elongation factor in *E. coli* (Schneider & Gibb, 1997). Since *Shigella* spp. have been demonstrated to be practically genetically indistinguishable from *E. coli* (Brenner *et al.*, 2005; Maheux *et al.*, 2009), these organisms are also detected by *tuf* PCR. Maheux and co-workers (2009) showed that targeting this gene resulted in the detection of 100% of *E. coli* and *Shigella* strains in the tested group, in comparison with the detection of between 97.5% and 100% of *E. coli* and 54.5% to 72.7% of *Shigella* strains with some *uidA* primers. When the specificity of targeting the *tuf* gene was evaluated, however, Maheux *et al.* (2009) found a 0.5% rate of false positives (0.0% for *uidA*) which was attributed to the erroneous detection of *Escherichia fergusonii*. Another alternative target gene which has been used to detect the presence of *E. coli* is the *mdh* gene, which is associated with catalysing the reversible oxidation reaction of malate to oxaloacetate by malate dehydrogenase (Park *et al.*, 1995). This gene has been utilised as a housekeeping gene for *E. coli* detection during a study to determine the occurrence of pathogenic strains of *E. coli* in water from wastewater treatment plants in South Africa (Omar & Barnard, 2010). In addition, *mdh* has also been used to assist in the identification of *E. coli* during bacterial source tracking in a constructed wetland (Martin, 2012). However, Omar & Barnard (2010) found that PCR inhibition occurred in DNA from wastewater samples, which resulted in a non-detection of the *mdh* gene. This problem could only be alleviated through a five-fold dilution of DNA samples, which is reported to adversely affect the specific detection of diarrhoeagenic *E. coli* (Omar & Barnard, 2010).

PCR also does not require cultivation before detection (Ashbolt *et al.*, 2001). Therefore, the method can detect organisms in the viable but non-culturable (VBNC) state (Girones *et al.*, 2010). In addition, molecular methods are sensitive, extremely specific and rapid (Girones *et al.*, 2010) after sample preparation, although it should be kept in mind that the PCR detection limit of a given target sequence may necessitate a pre-enrichment step, which would adversely affect the speed of the method. These methods hold another advantage over traditional detection methods: the distinction can be made between various diarrheagenic pathogenic strains and commensal *E. coli* through the use of multiplex PCR protocols (Omar & Barnard, 2010). The diarrheagenic pathogen strains of *E. coli* which are implicated in the contamination of surface water (Jagals *et al.*, 2006), namely EAEC, EHEC, EIEC, EPEC and ETEC, can be detected with the aid of distinctive genes present only in the particular pathotype. These pathotypes and their associated target genes are given in Table 2.8.

**Table 2.8** Diarrheagenic *E. coli* pathotypes and associated target genes used in PCR detection

<i>E. coli</i> pathotype	Target gene	Reference	Associated with <sup>†</sup>
EAEC	<i>eagg</i>	Kong <i>et al.</i> (2002)	Enteraggregative <i>E. coli</i>
EHEC	<i>eae</i>	López-Saucedo <i>et al.</i> (2003)	Intimin ( <i>E. coli</i> attaching and effacing)
	<i>stx1</i>	Moses <i>et al.</i> (2006)	Shiga-like toxin 1
	<i>stx2</i>	Moses <i>et al.</i> (2006)	Shiga-like toxin 2
EIEC	<i>ial</i>	Paton & Paton (1998); López-Saucedo <i>et al.</i> (2003)	Invasion-associated locus
EPEC	<i>eae</i>	López-Saucedo <i>et al.</i> (2003)	Intimin ( <i>E. coli</i> attaching and effacing)
ETEC	<i>lt</i>	Pass <i>et al.</i> (2000)	Heat-labile enterotoxin
	<i>st</i>	Pass <i>et al.</i> (2000)	Heat-stable enterotoxin

<sup>†</sup> Gassama-Sow *et al.*, 2004

Despite these advantages, many criticisms against these methods also exist: the PCR assay volume is very small (usually micro-litres) when compared to standard water sample volumes (typically 1 litre) (Ashbolt *et al.*, 2001) and water samples may contain substances that can interfere with or inhibit the PCR assay (Ashbolt *et al.*, 2001; Girones *et al.*, 2010). Also, PCR cannot distinguish between living and dead cells (Ashbolt *et al.*, 2001; Girones *et al.*, 2010) unless intercalating dyes such as ethidium or propidium monoazide (Girones *et al.*, 2010) are used. Cell numbers cannot be determined (Barnes, 2003) by ordinary PCR assays, but cells can be enumerated when quantitative PCR is used (Girones *et al.*, 2010). Direct PCR amplification is also difficult when the organism is present in low numbers (Girones *et al.*, 2010). Finally, DNA fingerprinting methods, such as RFLP, have low reproducibility (De Boer & Beumer, 1999). Despite these criticisms, molecular methods have established themselves as useful tools in the detection of organisms, especially those which cannot be detected by traditional methods.

## 2.7. CONCLUDING REMARKS

It is clear from the literature cited that rivers play an important role in South Africa, particularly in the irrigation of MPFs. Unfortunately, research has shown that there is an alarming level of microbial contamination in South African rivers. Some of these microbes can persist in water systems for prolonged periods of time even under adverse conditions, due to inherent endurance or through survival mechanisms such as biofilm or endospore formation. Furthermore, if these organisms are transferred to produce through contaminated irrigation water they have the ability to persist on surfaces or in the tissue of these products. This could lead to large-scale outbreaks due to the consumption of contaminated MPFs, as these pathogens are extremely harmful to humans.

To effectively rectify the microbiological state of South African rivers as well as implement preventative or corrective measures to ensure the safety of irrigated MPFs, it is necessary to monitor the contamination levels to ascertain the degree of improvement or deterioration of the water. For this purpose, *E. coli* is said to be the best indicator of faecal contamination even in the face of various suggestions of alternative indicators. However, it is uncertain whether the MTF method is sufficiently accurate and specific for the enumeration of *E. coli*. This leads to concerns that over- or under-estimations of the extent of faecal contamination can be made based on its results, and that all legal prosecution based on these has an unacceptable level of doubt. A variety of alternative methods have been developed and evaluated, but to date all have exhibited limitations and inaccuracies in their enumeration of *E. coli*.

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## CHAPTER 3

### BASELINE STUDY OF MICROBIOLOGICAL AND PHYSICO-CHEMICAL PARAMETERS OF THE UPPER BERG RIVER AND THEIR PREDICTIVE ABILITY FOR *E. COLI* COUNTS

#### 3.1. ABSTRACT

A thirteen month baseline study of three sites in the upper Berg River was done to determine the microbiological and physico-chemical state of this important source of irrigation water. Microbiological analysis of aerobic colony counts (ACC) and the enumeration of coliforms, *E. coli* and intestinal enterococci were used to determine general and faecal pollution levels in the river. Water from the river was also analysed for the presence of potential pathogens, i.e. *Listeria* spp., *Salmonella* spp., coagulase-positive staphylococci and sporeforming bacteria. Alkalinity, chemical oxygen demand (COD), electrical conductivity, pH and water temperature on-site were determined as physico-chemical measurements. Statistical correlations of selected measured microbiological and physico-chemical parameters with directly enumerated *E. coli* numbers were also determined.

Microbiological analyses of the entire sample set (three sampling sites) showed relatively low ACC (mean: 2 394 cfu.mL<sup>-1</sup>, median: 720 cfu.mL<sup>-1</sup>) and levels of intestinal enterococci (mean: 82 cfu.100 mL<sup>-1</sup>, median: 37 cfu.100 mL<sup>-1</sup>). However, the determined coliform (mean: 13 012 MPN.100 mL<sup>-1</sup>, median: 3 300 MPN.100 mL<sup>-1</sup>) and *E. coli* levels (mean: 2 696 MPN.100 mL<sup>-1</sup>, median: 330 MPN.100 mL<sup>-1</sup>) raise concern regarding the magnitude of the implied faecal pollution as well as the use of this water for irrigation of MPFs, since the *E. coli* levels exceeded the World Health Organization (WHO) guideline in 20.5% of cases. These *E. coli* levels as well the levels of other analysed bacteria generally tended to increase as sites progressed downstream, which was attributed to the accumulative effect of pollution from points upstream. *Listeria monocytogenes*, *Salmonella* spp. and other co-occurring potential pathogens, such as *Klebsiella pneumoniae*, were also detected in water from all three sites, which further exacerbates the risk to the health of consumers of minimally processed food (MPFs). Sporeforming bacteria were not detected once, and coagulase-positive staphylococci were only detected once at the downstream baseline site. Physico-chemical analyses indicated that while alkalinity and electrical conductivity measurements were within their respective regulations, COD levels were in excess of effluent discharge limits for South African listed rivers. In addition, the pH of the river water was found to be low in some instances (4.30 at its lowest reading), and did not fall within the Food and Agriculture Organization (FAO) guideline for irrigation water. The correlations of individual parameters which were evaluated against *E. coli* numbers were found to be poor and, in some instances, inverse. Furthermore, a regression analysis showed that the combined set of parameters accounted for less than half (adjusted multiple  $r^2=0.446$ ,  $p<0.01$ ) of the variation in *E. coli* numbers. Therefore,

none of the parameters which were investigated during this study are acceptable predictors which can be used instead of *E. coli* determinations.

### 3.2. INTRODUCTION

Rivers play an important role in the South African economy, with agriculture utilising around 52% of the water resources (Jackson *et al.*, 2009). Agricultural activities, in turn, contributed 2.2% of South Africa's gross domestic product in 2007 (Anon., 2008). These activities include the production fruit and vegetables which are minimally processed before being sold locally as well as exported. A considerable proportion of the production of export produce is concentrated in the Western Cape, where the Berg River is an important contributor of irrigation water. In addition, the banks and areas surrounding the Berg River are home to many towns and informal settlements. The inhabitation of land close to the river and consequent usage of water for domestic purposes by some communities, as well as recreational contact with the water, are additional reasons for ensuring that the water in the river is safe for contact and consumption.

Information on the quality of the water in the Berg River is sparse: one study evaluating microbiological enumeration techniques reported faecal coliform counts (Pulse *et al.*, 2007) which were frequently in violation of the WHO guideline for irrigation water used on crops likely to be eaten raw (WHO, 1989), with the highest counts occurring at a sampling site located adjacent to the Mbekweni informal settlement. Other workers reported that the levels of metal pollution in the Berg River are increasing (Jackson *et al.*, 2007) and also showed that fluctuations in inorganic nitrogen and phosphorous levels in the Berg River are in violation of South African water quality guidelines and that hypertrophic conditions are prevalent in this river (De Villiers, 2007). A study measuring the levels of endosulfan in water of the middle Berg River reported that the concentrations in the water were not very concerning but warranted routine monitoring (Dalvie *et al.*, 2003). In addition, local newspapers reported in 2005 that the European Union has threatened to remove South African export licences due to the levels of *Escherichia coli* in the Berg River (Cape Times Environmental Reporter, 2005). The Department of Water Affairs (DWA) of South Africa has also been monitoring the Berg River Water Management Area at various sampling points as part of their National Microbial Monitoring Programme (NMMP) (DWAF, 2002), but these results are not readily available. It is clear that a reliable long-term study needs to be undertaken to determine the quality of the upper Berg River, both in terms of chemical and microbiological parameters, to determine the risk that water from this river poses for both contact and irrigation of MPFs. This necessity has been addressed through the funding of a five-year nationwide research project (K5/1773), solicited by the Water Research Commission (WRC) and co-funded by the South African Department of Agriculture (DoA), focusing on the quality of South African rivers as irrigation sources for minimally processed foods.

When deciding on a sampling period, it is important to consider the fluctuations due to seasonal variations. These include elevated temperatures and increased sunlight radiation in summer months, which is said to reduce microbial populations in surface water (Curtis *et al.*, 1992; Maïga *et al.*, 2009; Schultz-Fademrecht *et al.*, 2008). Another factor which should be taken into account is larger volumes of water in winter months due to the high rainfall. This large volume of water is said to perform a “flushing” of microbes downstream in addition to its diluting effect, but also washes dry pollution on the riverbank into the river system (Dr. J.M. Barnes, Department of Community Health, Faculty of Health Sciences, Stellenbosch University, personal communication, 2010). This leads to a dualistic effect on the microbiological quality of the water during high rainfall periods.

Therefore, a sampling period to establish a credible baseline for this economically significant river should span no less than one year to account for all seasonal variations. Due to the large incidence of settlements close to the river in the area known as the upper Berg River catchment, it is expected that faecal pollution would be one of the main pollution problems. To determine whether this is the case, water is typically analysed for *Escherichia coli*, the globally accepted indicator of choice for faecal pollution (Edberg *et al.*, 2000). However, it has been the practise of some industries to employ predictive microbiology, that is, to measure chemical parameters instead of microbiological indicators for faecal pollution. This approach is mainly attributable to the more rapid results which are obtained by chemical measurements such as COD (results within a few hours) (Davis & McCuen, 2005), as well as the relative inexpensiveness when compared to microbiological analyses.

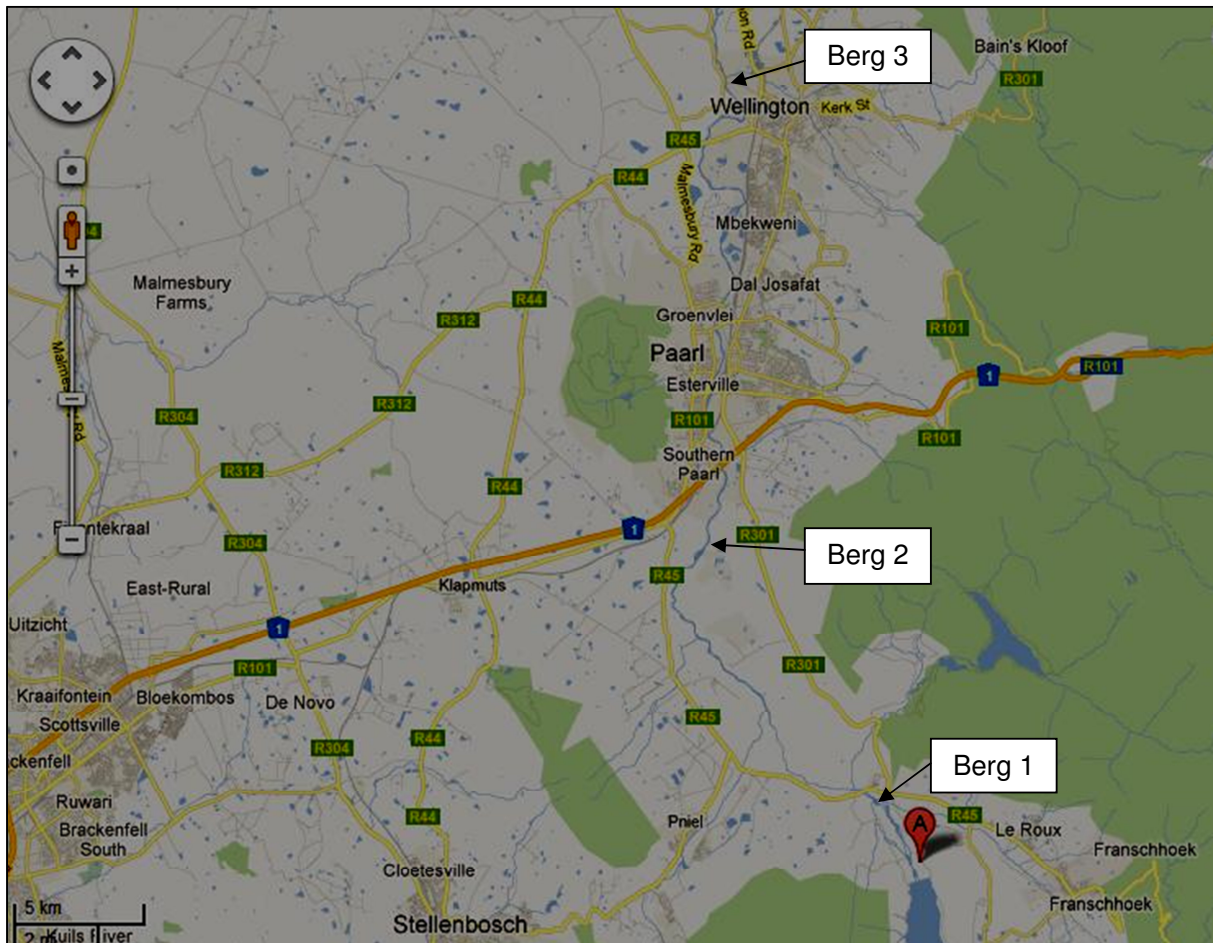
The aims of this study are: (1) to determine the microbiological and chemical quality of samples from the upper Berg River to determine the microbiological and physico-chemical baseline quality and (2) to determine the degree to which alternative microbiological, physical and chemical parameters can be used to predict *E. coli* numbers in the upper Berg River.

### **3.3. MATERIALS AND METHODS**

#### **3.3.1. Site selection**

For the baseline determination of the quality of the upper Berg River, three sites were selected. The upstream baseline site (Berg 1) was located approximately 7 km downstream from the Berg River Dam, after confluence with the Franschhoek/Stiebeuel tributary which passes through the town and informal settlements of Franschhoek. The second site, Berg 2, was located approximately 20 km (by road) from Berg 1 and downstream from the confluence with the Wemmershoek River tributary. This site was directly downstream from a commercial farm which produces tomatoes with water abstracted from the river which is chlorinated before use. The third site (Berg 3), the downstream baseline site, was located approximately 30 km (by road) from Berg

2. This sampling site was situated 500 m downstream of the Lady Loch Bridge, roughly 1.5 km outside of Wellington, and was the site for sampling river water which had passed through Paarl and its informal settlements, Wellington, as well as the wastewater treatment works of both towns. The geographical locations of these sites are indicated in Figure 3.1.



**Figure 3.1** Geographical locations of three sampling sites in the upper Berg River (Map data ©2012 AfriGIS (Pty) Ltd, Google)

### 3.3.2. Sample collection

Samples were collected monthly, from March 31<sup>st</sup> 2008 to March 16<sup>th</sup> 2009 from the three selected sites, and were drawn on the morning of the same day in ascending numerical order, with Berg 1 sampled first and Berg 3 sampled last. One litre of river water was drawn in a sterile container using the guidelines as set out by the Standard Methods for the Examination of Water and Wastewater (2005). Samples were placed on ice, transported to the laboratory, and analysed within four hours.



### 3.3.3. Microbiological parameters

Before analyses were performed, the sampling container was vigorously shaken to ensure a homogenous and representative sample of water was used for analysis. The analyses for ACC as well as aerobic and anaerobic sporeformers were selected to represent the concentration of culturable bacteria, to ascertain the general microbiological quality of the water. The enumerations for intestinal enterococci, coliforms and *E. coli* were selected as an indication of faecal contamination. For the detection of specific pathogens, *Listeria monocytogenes*, *Salmonella* spp. and coagulase-positive staphylococci were selected as indicators of potential agents of gastrointestinal disease. *Listeria* spp. are known to grow at temperatures as low as 4°C, which is the typical refrigeration temperature for storage of many MPFs.

#### ACC

The enumeration of aerobic bacteria was performed according to the South African Bureau of Standards (SABS) method SABS 4833 (SABS, 1991), and results were expressed as the number of colony forming units per mL of sample. Culturing was done on plate count agar (PCA, Biolab, Merck, Wadeville, South Africa) using the pour-plate method.

#### Aerobic and anaerobic sporeformers

The enumeration of aerobic and anaerobic sporeformers was performed according to method MFLP-44 (Austin, 1998) as prescribed by Health Canada. Sporeforming bacteria were cultured on tryptose soy agar (TSA, Oxoid, Basingstoke, Hampshire, UK) using the pour-plate method for 1 mL of undiluted sample and serial dilutions up to  $10^{-7}$ . Results were expressed as the number of colony forming units per mL of sample.

#### Coliforms and *E. coli*

Serial dilutions prepared up to  $10^{-7}$  were analysed using five MTF tubes per dilution (including undiluted double and single strength samples). Coliforms and *E. coli* were enumerated using the MFHBP-19 method outlined by Health Canada (Christensen *et al.*, 2002) with lauryl sulphate tryptose (LST) and brilliant green lactose bile (BGLB) broths (Oxoid, Basingstoke, Hampshire, UK) enumerating presumptive and confirmed coliforms, respectively. The addition of 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG, Oxoid, Basingstoke, Hampshire, UK) to the *E. coli* (EC; Oxoid, Basingstoke, Hampshire, UK) broth step enabled the direct enumeration of *E. coli* instead of faecal coliforms. Enumeration of coliforms and *E. coli* was done using the De Mans most probable number (MPN) table (Standard Methods for the Examination of Water and Wastewater, 1995), and results were expressed as coliforms or *E. coli* MPN.100 mL<sup>-1</sup>. Verification of *E. coli* enumerations in EC broth with MUG was done on Levine-eosin methylene blue (L-EMB) agar (Oxoid, Basingstoke, Hampshire, UK).



### Intestinal enterococci

The enumeration of intestinal enterococci was done according to the South African National Standards (SANS) method 7899-2 (SABS, 2004), by incubating a 0.45 µm membrane filter on Slanetz & Bartley (Oxoid, Basingstoke, Hampshire, UK) and bile aesculin (Oxoid, Basingstoke, Hampshire, UK) agars. Results of intestinal enterococci enumerations were expressed as colony forming units per 100 mL of filtered sample.

### *Listeria monocytogenes*

The presence or absence of *L. monocytogenes* in the water samples was determined using the horizontal detection method SABS 11290-1 for *L. monocytogenes* (SABS, 1996). This method requires the use of PALCAM agar and Agar Listeria (according to Ottoviani and Agosti) as solid media. Agar Listeria could not be locally obtained, and Oxford agar (Oxoid, Basingstoke, Hampshire, UK) was used as replacement with PALCAM agar (Oxoid, Basingstoke, Hampshire, UK). Oxford agar is recommended for the detection of *L. monocytogenes*, along with PALCAM agar, by the International Organization for Standardization (ISO) (Pinto *et al.*, 2001). Primary enrichment of 1 mL of sample was done in half-Fraser broth, and was followed by secondary enrichment in full-Fraser broth. After the enrichment steps, the presence or absence of listeriae was determined through spread-plating on Oxford and PALCAM agars. Colonies of *Listeria* were visually identified with the aid of the Merck Microbiology Manual (Merck, 2007).

### *Salmonella* spp.

The presence or absence of *Salmonella* spp. was determined using the SABS 6579 method (SABS, 1993). Xylose lysine desoxycholate (XLD, Oxoid, Basingstoke, Hampshire, UK) agar was used for detection after primary enrichment of 25 mL of sample in 225 mL buffered peptone water (BPW, Oxoid, Basingstoke, Hampshire, UK), and secondary enrichment in selenite cysteine (SC) broth (Oxoid, Basingstoke, Hampshire, UK) and Rappaport-Vassiliadis (RV) broth (Oxoid, Basingstoke, Hampshire, UK). Only presence or absence was reported after spread-plating on XLD agar. *Salmonella* colonies were identified visually using Merck's Microbiology Manual (Merck, 2007).

### Coagulase-positive staphylococci

*Staphylococcus aureus* and the other five members of the coagulase-positive group (*S. delphini*, *S. hyicus*, *S. intermedius*, *S. lutrae*, *S. pseudintermedius* and *S. schleiferi* subsp. *coagulans*) of staphylococci were enumerated using the SABS 6888-1 method (SABS, 1999). Coagulase-positive staphylococci were cultured on Baird-Parker agar (Oxoid, Basingstoke, Hampshire, UK) with egg yolk tellurite emulsion (Oxoid, Basingstoke, Hampshire, UK) at 5.0% v/v using the spread-

plate method for 1 mL of undiluted sample and serial dilutions up to  $10^{-7}$ . Results were expressed as colony forming units per mL of sample.

#### Identification of atypical colonies

Atypical colonies on *Salmonella*, *Listeria* and *E. coli* selective media plates were identified visually with the aid of Merck's Microbiology Manual (Merck, 2007). Atypical colonies were randomly selected to represent all atypical types on a given medium and purified by streaking on nutrient agar (16% w/v nutrient broth (Biolab) and 12% w/v bacteriological agar (Biolab)). Purified colonies were Gram stained and observed under a light microscope (Nikon Eclipse E400, Nikon Corporation, Tokyo, Japan). Catalase slide method test (Reiner, 2010) and oxidase filter paper method test with 1% Kovács reagent (Shields & Cathcart, 2010) were performed on the purified organism.

Based on morphological appearance as well as catalase and oxidase reactions, the biochemical profiles of the organisms were determined using either Analytical Profile Index (API) 20E or the API *Listeria* strips, and were subsequently identified by APIweb™ software (bioMérieux SA, Marcy l'Étoile, Rhône, France).

#### 3.3.4. Physical and chemical parameters

Water temperature was measured on-site, and the alkalinity, COD, electrical conductivity and pH of the water samples were measured in the laboratory.

##### Temperature

The temperature of the river water was measured on-site using a handheld thermometer (Thermometer 638Pt, Crison Instruments SA, Barcelona, Spain). The probe was submerged to a depth of approximately 20 cm and the temperature recorded.

##### Alkalinity

The alkalinity of the water samples was determined using the method described in Standard Methods for the Examination of Water and Wastewater (2005). Results were expressed as mg  $\text{CaCO}_3 \cdot \text{L}^{-1}$ .

##### COD

The COD values were determined using the method in Standard Methods for the Examination of Water and Wastewater (2005). Digestion was performed using a block digester at 150 °C (Hach Company, Loveland, Colorado, USA) and absorbance values were read with a direct reading spectrophotometer (HACH DR/2000, Hach Company, Loveland, Colorado, USA) at 585 nm. Absorbance values were converted to COD values, which were expressed as mg  $\text{O}_2 \cdot \text{L}^{-1}$ .

### Conductivity

The conductivity of water samples was determined according to the method outlined in Standard Methods for the Examination of Water and Wastewater (2005). A conductivity meter (HI 8733, Conductivity meter, Hanna Instruments, Woonsocket, Rhode Island, USA) was used for the measurements. Results were expressed as  $\text{mS.m}^{-1}$ .

### pH

The pH of water samples were determined using a standard laboratory pH meter (Microprocessor pH-meter pH 320, WTW, Weilheim, Germany) at ambient temperature.

#### **3.3.5. Statistical correlation of quantified parameters with *E. coli* concentration**

The accuracy of ACC, coliform counts, river temperature, COD values, electrical conductivity values and pH as predictors of *E. coli* numbers were assessed using correlation statistics. Due to the microbiological component in these analyses, calculation of the Spearman rank correlation coefficient was favoured over normal Pearson correlations due to the robustness of Spearman correlation against outlier values (Bin Abdullah, 1990).

A regression analysis with *E. coli* as dependent variable was also performed to determine the extent to which the collective set of measurements could account for variation in *E. coli* numbers. Finally, a Durbin-Watson serial correlation for all analysed parameters was conducted.

The data set for statistical analyses was increased through the inclusion of a second sampling season (from April 2009 until May 2010). Statistical analyses were conducted by Prof. M. Kidd at the Center for Statistical Consultation, Stellenbosch University.

## **3.4. RESULTS AND DISCUSSION**

### **3.4.1. Microbiological parameters**

#### ACC

The ACC results are reported in Table 3.1. Non-detection events were taken as  $0 \text{ cfu.mL}^{-1}$  for the purposes of the discussion.

**Table 3.1** Aerobic colony counts for river water samples from three Berg River sampling points

Sampling date	Aerobic colony count (cfu.mL <sup>-1</sup> )		
	Berg 1	Berg 2	Berg 3
03/2008	310	<10	7 800
04/2008	<10	1 060	1 770
05/2008	<10	<10	<10
06/2008	790	<10	<10
07/2008 <sup>‡</sup>	700	<10	2 450
08/2008	650	670	8 200
09/2008	600	750	2 390
10/2008	8 700	11 800	14 700
11/2008 <sup>‡</sup>	720	4 700	14 800
12/2008	810	2 580	1 410
01/2009	<10	400	440
02/2009	<10	580	1 110
03/2009	930	1 150	410
<b>Site mean</b>	1 579	2 632	5 044
<b>Site median</b>	650	670	1 770

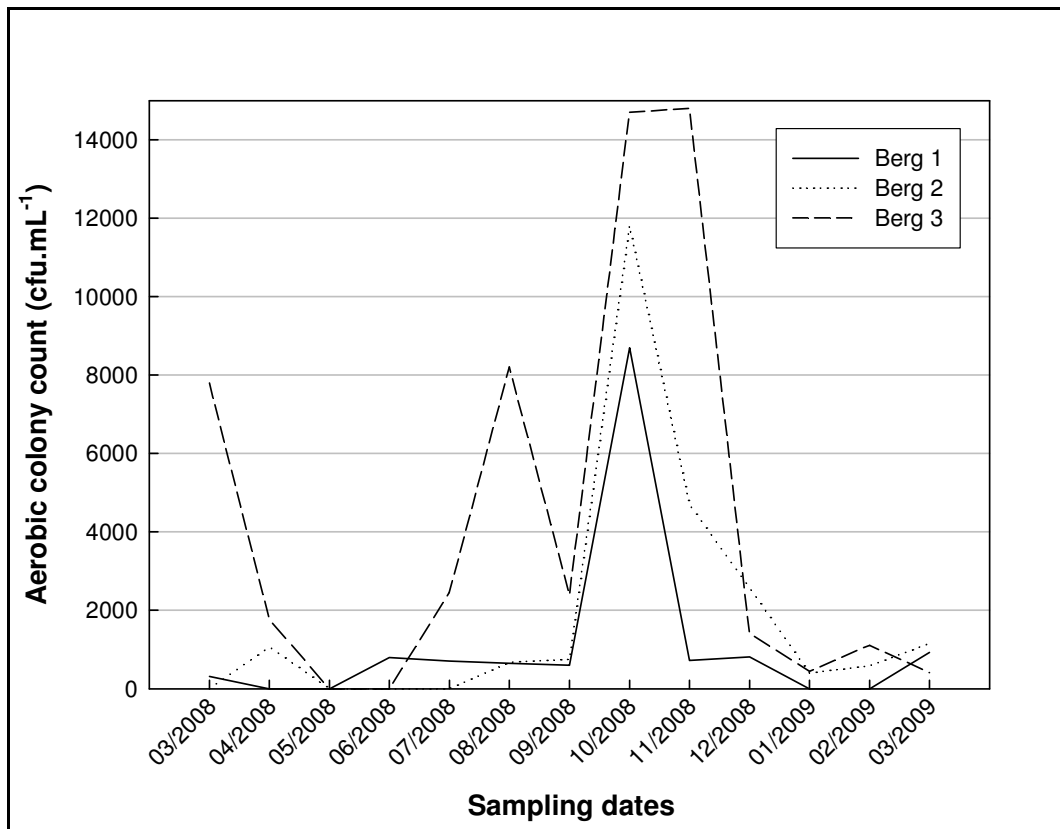
<sup>‡</sup>Sampling after rainfall

ND - none detected

The ACC results ranged from 310 (Berg 1, 03/2008) to 14 800 cfu.mL<sup>-1</sup> (Berg 3, 11/2008). When examining the aerobic colony counts for the three sites per sampling date, it is clear that the number of aerobic bacteria in the water is higher, in most cases, at sites located further downstream. This is especially true when comparing the values of Berg 3 with those of Berg 2, where an increase as large as 10 100 cfu.mL<sup>-1</sup> was observed on 11/2008. Only in two cases (12/2008 and 03/2009) was this pattern not followed. The increase in values from Berg 1 to Berg 2 were less marked, with the highest increase being 3 980 cfu.mL<sup>-1</sup>. There were, however, three cases (23.1%) for Berg 2 where no aerobic bacteria were detected, while high counts were detected for Berg 1. The general pattern of increasing aerobic colony counts downstream can also be observed in the site mean values, which increase slightly from Berg 1 to Berg 2, and more than two-fold from Berg 2 to Berg 3.

The aerobic colony counts which are reported here indicate that the general microbiological conditions of the river are deteriorating as water progresses downstream. This is in keeping with the theory that identified river-proximate settlements, agricultural activities and industries as likely contributors of microbiological pollution of the upper Berg River. The unexpected higher upstream values which occasionally occurred are most probably due to an exceptional point-source pollution event, and non-detection of bacteria further downstream in these cases could indicate that the pollution level was low enough to be diluted by the volume of water flowing between the two sites.

In terms of seasonal variation, the highest aerobic colony counts for all sites occurred around October of 2008. To better visualise the variation in counts across the 13 months, Fig. 3.2 shows the line plots for all three sites.



**Figure 3.2** ACC for river water samples from all three sites for the 13 months

The results for Berg 3 show that a spike in ACC was detected for August 2008. Since no similar trend can be observed for the two sites which occur upstream, this spike is most probably attributable to a point-source pollution event. The towns of Paarl and Wellington, as well as their informal settlements and wastewater treatment works, are located just upstream from this site and would be the first suspected perpetrators. However, the corresponding coliform and *E. coli* counts for this date were not noticeably higher than the preceding or following months, which decrease the likelihood that this spike in ACC was due to faecal pollution. High counts were also observed for all sites in 10/2008. In contrast, however, this spike is probably indicative of a large point-source pollution event high upstream since the corresponding coliform and *E. coli* counts for this month also showed spikes. Since this dramatic increase in microbiological indicators was observable for all three sites, this point-source pollution event must have occurred above the first sampling site near the town of Franschhoek and its wastewater treatment works. However, the increase in numbers for this spike as the sites progress downstream is evidence that bacterial numbers were also fortified by pollution downstream from the first sampling site (Berg 1).

### Aerobic and anaerobic sporeformers

No aerobic or anaerobic sporeformers were detected (minimum detection limit <100/mL) for any one of the three sites during the entire sampling period which presumably indicates the absence of sporeforming genera such as *Bacillus* and *Clostridium*. However, the non-detection of these organisms may also be attributable to low numbers in the water which could not be detected in the small volumes which were tested.

### Coliforms

The coliform counts and mean coliform counts for each site are given in Table 3.2.

**Table 3.2** Coliform counts for the river water samples from three sampling points

Sampling date	Coliforms (MPN.100 mL <sup>-1</sup> )		
	Berg 1	Berg 2	Berg 3
03/2008	790	580	110 000
04/2008	330	4 900	3 300
05/2008	49	490	700
06/2008	3 500	1 500	31 000
07/2008 <sup>‡</sup>	3 300	13 000	79 000
08/2008	13 000	9 500	23 000
09/2008	1 300	2 200	13 000
10/2008	49 000	7 000	49 000
11/2008 <sup>‡</sup>	17 000	7 900	23 000
12/2008	3 300	1 300	23 000
01/2009	23	1 300	1 700
02/2009	280	1 800	7 000
03/2009	46	130	230
<b>Site mean</b>	7 071	3 969	27 995
<b>Site median</b>	1 300	1 800	23 000

<sup>‡</sup>Sampling after rainfall

The coliform values for Berg 2 and Berg 3 once again show a trend towards higher coliform numbers, with one exception (04/2008). Interestingly, the values for Berg 2 were considerably lower (at 95% confidence interval) than that of Berg 1 in three cases (23.1%). Additionally, the mean value for Berg 2 was nearly half of the mean obtained for Berg 1. The mean for Berg 3 was, however, markedly higher than that of the other two sites.

These observations are further indication that human settlements and their activities contribute to pollution of downstream waters. In addition, the proportion of instances where

coliform values were lower for Berg 2 than Berg 1 suggests that the absence of settlements in the area surrounding Berg 2 contributed to the lowered coliform counts, since die-off of bacteria which were deposited upstream occurred without replenishment through pollution around Berg 2. This, by extension, indicates that a major contributor of faecal pollution in this region could be human settlements.

The increase in coliform counts over the seasons follows the same trend as that of aerobic colony counts. There is a marked increase in coliform numbers for all sites in 06/2008, with a dramatic decrease from 01/2009 (except in the case of Berg 2, where counts only decreased in 03/2009). This trend suggests that rainfall events increase the pollution of the river through the washing of riverbank pollution into the system and that the dilution effect plays a negligible role in decreasing this pollution.

### *E. coli*

The *E. coli* values for each site are given in Table 3.3 and as Fig. 3.3, the latter along with the WHO's guideline for irrigation water used for the irrigation of crops likely to be eaten raw, set at 1 000 thermotolerant or faecal coliforms MPN.100 mL<sup>-1</sup> (WHO, 1989). Since thermotolerant coliforms were not directly enumerated due to the presence of MUG in EC broth, the guideline was applied to the *E. coli* results. *E. coli* forms part of the thermotolerant coliform group which encompasses species within the genera *Citrobacter*, *Enterobacter*, *Escherichia* and *Klebsiella* (Leclerc *et al.*, 2001), and the exceeding of 1 000 MPN.mL<sup>-1</sup> by one species of the thermotolerant coliform group implies greater transgression of the WHO guideline by the entire group.

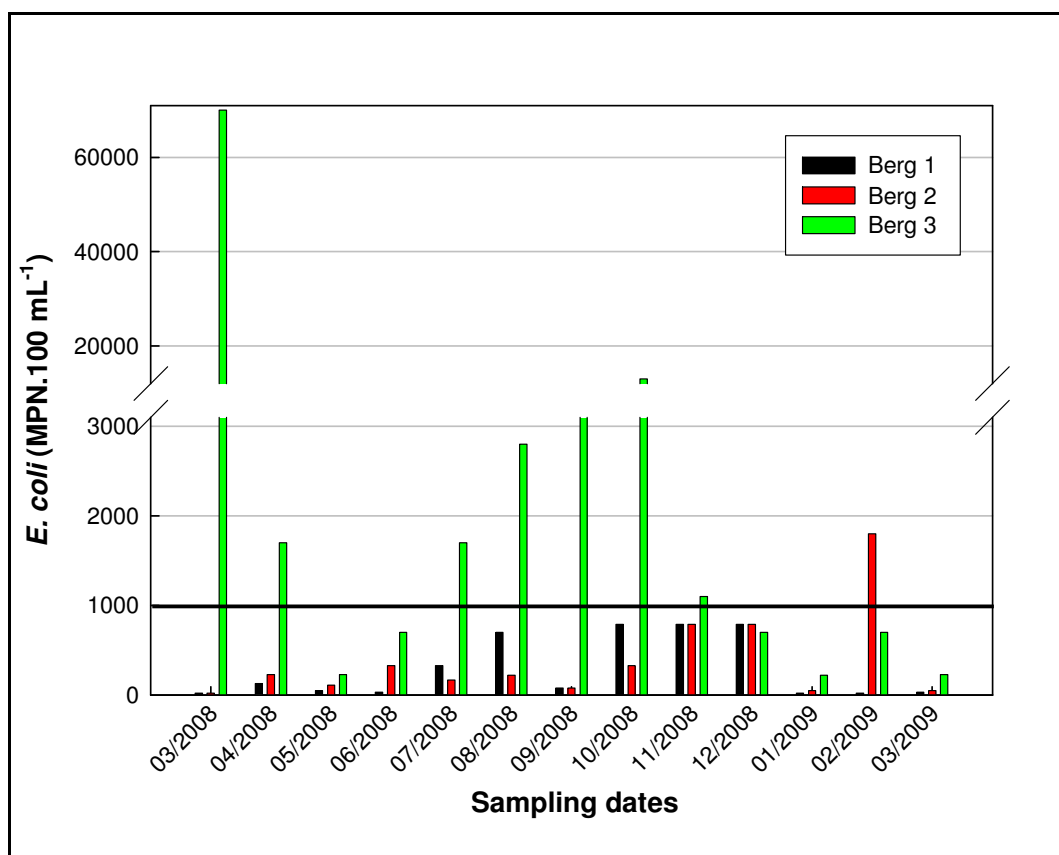
It can be seen in Fig. 3.3 that river water from Berg 3 transgressed the WHO guideline in seven instances, while water from Berg 2 sampled in February of 2009 also did not conform to this guideline value. The transgressions of Berg 3 do not bode well for farmers using the water for the irrigation of their produce, and can pose a risk to the health of local and international consumers as well as jeopardise export agreements with countries requiring conformance with WHO guidelines. The 03/2008 value for this site is, at 70 000 *E. coli* MPN.100 mL<sup>-1</sup>, a point of concern for the health of both consumers and individuals coming into contact with this water. Such high numbers of the indicator bacteria is indicative of considerable faecal pollution upstream, most likely from settlements in Wellington or the Wellington wastewater treatment works.

**Table 3.3** *E. coli* counts for the river water samples from three sampling points

Sampling date	<i>E. coli</i> (MPN.100 mL <sup>-1</sup> )		
	Berg 1	Berg 2	Berg 3
03/2008	79	79	49 000
04/2008	130	230	790
<b>Table 3.3 continued</b>			



Table 3.3 continued			
Sampling date	<i>E. coli</i> (MPN.100 mL <sup>-1</sup> )		
	Berg 1	Berg 2	Berg 3
05/2008	49	110	230
06/2008	48	170	64
07/2008 <sup>†</sup>	40	22	1 300
08/2008	700	220	2 800
09/2008	79	79	440
10/2008	32	40	1 700
11/2008 <sup>†</sup>	37	29	1 100
12/2008	280	790	330
01/2009	7.8	49	220
02/2009	23	1 400	220
03/2009	23	49	34
<b>Site mean</b>	<b>118</b>	<b>251</b>	<b>4479</b>
<b>Site median</b>	<b>48</b>	<b>79</b>	<b>440</b>



**Figure 3.3.** *E. coli* values for samples from the three sites and their conformance to WHO guideline for the irrigation of crops likely to be eaten raw. The WHO guideline value is shown by the heavy black horizontal line

The trend for increased *E. coli* numbers for downstream sites can again be observed in Fig. 3.3. In addition, there are several instances where values at Berg 1 were higher than those at Berg 2, which corresponds to the trends observed for both ACC and coliform values. Interestingly, the *E. coli* value for Berg 2 was the highest of all the sites in 02/2009. This phenomenon is presumably attributable to an exceptional point-source pollution event.

In terms of seasonal variation, the increase in *E. coli* values during the period from 06/2008 to 11/2008 once again agrees with the trends observed for aerobic colony counts and coliform numbers for the three sites. The observation that *E. coli* numbers also follow this trend is evidence in support of the suspicion that faecal pollution is the major contributor of pollution in the upper Berg River.

#### Intestinal enterococci

The intestinal enterococci counts and calculated mean counts for each site are given in Table 3.4.

**Table 3.4** Intestinal enterococci counts for river water samples from three sampling points

Sampling date	Intestinal enterococci count (cfu.100 mL <sup>-1</sup> )		
	Berg 1	Berg 2	Berg 3
03/2008	1	16	3
04/2008	ND	48	135
05/2008	ND	89	ND
06/2008	1	82	145
07/2008 <sup>†</sup>	75	58	4
08/2008	1	7	52
09/2008	21	ND	150
10/2008	TNTC	2	TNTC
11/2008 <sup>†</sup>	109	TNTC	TNTC
12/2008	1	9	12
01/2009	4	TNTC	37
02/2009	54	TNTC	14
03/2009	23	TNTC	TNTC
<b>Site mean</b>	24	35	55
<b>Site median</b>	3	16	26

<sup>†</sup>Sampling after rainfall

ND - none detected

The results reported in Table 3.4 are somewhat surprising when comparing them with the results in Table 3.2 and Fig. 3.3, since the intestinal enterococci group is recommended as an indicator of faecal pollution (Ashbolt *et al.*, 2001). These results do not agree well with those obtained for coliforms or *E. coli*. When the mean counts for intestinal enterococci in Table 3.4 are

compared with the mean counts for *E. coli* as given in Fig. 3.3, it is clear that the trend for increasing *E. coli* numbers downstream was not reflected in intestinal enterococci counts. In addition, the seasonal trend which could be seen in aerobic colony counts, coliform and *E. coli* counts was not followed by the intestinal enterococci counts. It is possible that these organisms were simply absent from the water. This would suggest that the measurement of this parameter may not be the most effective in the monitoring faecal pollution in the upper Berg River.

The presence of these organisms in the water should, however, be cause for disquiet. The *Enterococcus* genus is known for a high intrinsic level of antibiotic resistance, and some of these organisms can cause clinical infections ranging from urinary tract infection (Bitsori *et al.*, 2005) to meningitis or ventriculitis (Elvy *et al.*, 2008). The possibility of such organisms occurring in the river is highly undesirable for water which is used for the irrigation of fresh produce, as well as for domestic and recreational contact.

### *Listeria monocytogenes*

The results from the *L. monocytogenes* analysis for the river water samples after the primary and secondary enrichment step are given in Tables 3.5 and 3.6, respectively. Although the method which was used for the detection was directed towards the determination of *L. monocytogenes*, other members of the genus were also observed and recorded.

**Table 3.5** Results for *L. monocytogenes* analyses from three sampling sites after primary enrichment

Sampling date	Presence or absence of <i>Listeria</i> in half Fraser broth		
	Berg 1	Berg 2	Berg 3
03/2008	<i>L. innocua</i>	<i>L. innocua</i> , <i>L. monocytogenes</i>	<i>L. grayi</i>
04/2008	<i>L. grayi</i>	<i>L. grayi</i>	<i>L. grayi</i> , <i>L. innocua</i>
05/2008	<i>L. grayi</i>	ND	ND
06/2008	ND	ND	<i>L. innocua</i> , <i>L. monocytogenes</i>
07/2008 <sup>+</sup>	ND	<i>L. innocua</i>	<i>L. monocytogenes</i>
08/2008	ND	ND	ND
09/2008	<i>L. innocua</i>	ND	ND
10/2008	ND	ND	ND
11/2008 <sup>+</sup>	<i>L. innocua</i>	ND	<i>L. grayi</i>
12/2008	<i>L. innocua</i>	ND	ND
01/2009	<i>L. innocua</i>	<i>L. innocua</i>	<i>L. innocua</i>
02/2009	ND	ND	ND
03/2009	ND	<i>L. ivanovii</i>	<i>L. monocytogenes</i>

ND - none detected

<sup>+</sup>Sampling after rainfall

**Table 3.6** Results for *L. monocytogenes* analyses from three sampling sites after secondary enrichment

Sampling date	Presence or absence of <i>Listeria</i> in full Fraser broth		
	Berg 1	Berg 2	Berg 3
03/2008	<i>L. innocua</i>	<i>L. innocua</i> , <i>L. monocytogenes</i>	<i>L. grayi</i>
04/2008	<i>L. grayi</i> , <i>L. innocua</i>	<i>L. grayi</i>	ND
05/2008	ND	ND	ND
06/2008	ND	ND	ND
07/2008 <sup>†</sup>	ND	<i>L. innocua</i>	ND
08/2008	ND	ND	ND

Sampling date	Presence or absence of <i>Listeria</i> in full Fraser broth		
	Berg 1	Berg 2	Berg 3
09/2008	<i>L. innocua</i> , <i>L. monocytogenes</i>	<i>L. innocua</i> , <i>L. monocytogenes</i>	<i>L. innocua</i> , <i>L. monocytogenes</i>
10/2008	ND	ND	ND
11/2008 <sup>†</sup>	<i>L. innocua</i>	<i>L. monocytogenes</i>	<i>L. grayi</i>
12/2008	ND	ND	ND
01/2009	ND	<i>L. innocua</i>	<i>L. innocua</i>
02/2009	ND	ND	ND
03/2009	ND	ND	<i>L. innocua</i>

ND - none detected

<sup>†</sup>Sampling after rainfall

It is clear from Tables 3.5 and 3.6 that *Listeria* spp. occurred frequently in the waters of the upper Berg River. In addition, it appears that secondary enrichment favours the growth of *L. monocytogenes* when the proportion of detection events with species other than *L. monocytogenes* is compared for primary (81.8%) and secondary (75.0%) enrichment. Since the method used for the detection is specifically for the detection of *L. monocytogenes*, this is not surprising. This does, however, imply that other species were coincidentally detected here and were possibly under-detected relative to their abundance in the water.

The occurrence of *L. monocytogenes*, and any of the other *Listeria* species, did not follow the seasonal increase which was observed in winter and spring with other microbiological measurements. The incidence of *L. monocytogenes* did increase during the winter months, but this increase was not marked. This increase may be attributable to larger volumes of water in the river which causes plants and plant matter, known sources of listeriae, to be present in elevated amounts in the water. Similarly, it is extremely difficult to distinguish any pattern in progression downstream since these organisms were not quantified. The frequency of detection in Berg 3 does seem to be somewhat higher than for the other two sites, but the results are not compelling enough to justify any concrete conclusions.

Despite not being able to draw conclusions relating to seasonal variation patterns or the impact of pollution on downstream numbers, the results are still cause for concern. The presence of *L. monocytogenes* in food or water, which comes into contact with humans poses a significant health risk for listeriosis and listerial gastroenteritis (FDA, 2003). This food-borne pathogen is capable of growth at 4°C, the refrigeration temperature used for many MPFs, and can cause gastroenteritis, septicaemia and meningitis (Farber & Peterkin, 1991). Although this organism is present in a wide variety of environments such as animal and bird faeces, soil and plant matter (Freitag *et al.*, 2009; Gilbreth *et al.*, 2004), it is also found in the gut of healthy humans. Therefore, its presence, in conjunction with high levels of *E. coli*, could be indicative of faecal pollution in the upper Berg River.

### *Salmonella* spp.

The results of the detection for the presence of *Salmonella* spp. from the river water samples after the secondary enrichment steps in Rappaport-Vassiliadis (RV) and selenite cysteine (SC) are given in Tables 3.7 and 3.8, respectively. These secondary enrichment steps in combination with the primary enrichment in buffered peptone water were directed, according to the method, towards the exclusive determination of *Salmonella* spp. However, other *Enterobacteriaceae* genera, as well as members of *Aeromonas* and *Pseudomonas*, were also frequently identified, using the Merck Microbiology Manual (Merck, 2007), from the agar during these analyses.

It is clear from the results in Tables 3.7 and 3.8 that the SC broth produced a higher detection frequency than RV broth for *Salmonella* spp., based on the combined results; with an additional 20.5% (from 33.3% for RV broth to 53.8% for SC broth) of water samples indicating the presence of members of this genus. It is for this reason that the combined results from SC and RV are typically used to determine the presence of *Salmonella*, as both methods underestimate the occurrence of this important potential pathogen.

**Table 3.7** Results for *Salmonella* spp. analyses in river water from three sampling sites after secondary enrichment in RV broth

Sampling date	Presence or absence of <i>Salmonella</i> spp. in RV broth		
	Berg 1	Berg 2	Berg 3
03/2008	<i>Enterobacter</i>	<i>Enterobacter</i> , <i>Klebsiella</i>	<i>Citrobacter</i> , <b><i>Salmonella enteritidis</i></b>
04/2008	<i>Enterobacter</i>	<i>Enterobacter</i>	<i>Enterobacter</i> , <b><i>Salmonella enteritidis</i></b>
05/2008	<i>Klebsiella</i>	<i>Klebsiella</i> )	<i>Klebsiella</i> , <i>Serratia</i>
06/2008	<i>Klebsiella</i>	<i>Klebsiella</i> , <i>Serratia</i>	<i>Klebsiella</i> , <b><i>Salmonella enteritidis</i></b>
07/2008 <sup>‡</sup>	<i>Klebsiella</i>	<i>Klebsiella</i>	<i>Klebsiella</i> , <b><i>Salmonella enteritidis</i></b>
08/2008	<i>Klebsiella</i>	<i>Klebsiella</i>	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Klebsiella</i>
09/2008	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Klebsiella</i>	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Citrobacter</i> , <i>Klebsiella</i> , <b><i>Salmonella</i></b>	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Citrobacter</i> , <i>Klebsiella</i> , <b><i>Salmonella</i></b> , <i>Serratia</i>
10/2008	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Klebsiella</i> , <b><i>Salmonella enteritidis</i></b>	<i>Klebsiella</i>	<i>Klebsiella</i>
11/2008 <sup>‡</sup>	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Klebsiella</i>	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Klebsiella</i>	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Klebsiella</i>
12/2008	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Klebsiella</i>	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Klebsiella</i>	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Klebsiella</i> , <b><i>Salmonella</i></b>
01/2009	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Klebsiella</i> , <b><i>Salmonella enteritidis</i></b>	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Klebsiella</i>	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <b><i>Salmonella enteritidis</i></b>
02/2009	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Proteus mirabilis</i> / <b><i>Salmonella enteritidis</i></b>	ND	ND
03/2009	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <b><i>Salmonella enteritidis</i></b>	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Klebsiella</i>	<i>Aeromonas</i> / <i>Enterob.</i> / <i>E. coli</i> , <i>Citrobacter</i> , <b><i>Salmonella enteritidis</i></b>

ND - none detected

<sup>‡</sup>Sampling after rainfall**Table 3.8** Results for *Salmonella* spp. analyses in river water from three sampling sites after secondary enrichment in SC broth

Sampling date	Presence or absence of <i>Salmonella</i> spp. in SC broth		
	Berg 1	Berg 2	Berg 3
03/2008	<i>Klebsiella pneumoniae</i> , <b><i>Salmonella</i></b>	<i>Enterobacter</i> , <b><i>Salmonella</i></b>	<i>Citrobacter</i> , <i>Klebsiella</i> , <b><i>Salmonella</i></b>
04/2008	<b><i>Salmonella enteritidis</i></b> , <i>Serratia</i>	<i>Enterobacter</i> , <i>Serratia</i>	<b><i>Salmonella</i></b>
05/2008	<i>Hafnia</i> / <i>Serratia</i>	<i>Klebsiella</i>	<i>Citrobacter</i> , <i>Klebsiella</i>

Table 3.8 continued

Table 3.8 continued

Sampling date	Presence or absence of <i>Salmonella</i> spp. in SC broth		
	Berg 1	Berg 2	Berg 3
06/2008	<i>Aeromonas/Enterob./ E. coli, Klebsiella</i>	<i>Klebsiella</i>	<i>Aeromonas/Enterob./ E. coli, Klebsiella, Salmonella enteritidis</i>
07/2008 <sup>‡</sup>	<i>Klebsiella, Salmonella enteritidis, Hafnia/Serratia</i>	<i>Klebsiella, Salmonella enteritidis</i>	<i>Citrobacter, Salmonella, Serratia</i>
08/2008	<i>Aeromonas/Enterob./ E. coli, Klebsiella</i>	<i>Aeromonas/Enterob./ E. coli, Klebsiella</i>	<i>Aeromonas/Enterob./ E. coli, Klebsiella, Salmonella enteritidis</i>
09/2008	<i>Aeromonas/Enterob./ E. coli, Citrobacter, Salmonella</i>	<i>Aeromonas/Enterob./ E. coli, Citrobacter, Salmonella</i>	<i>Aeromonas/Enterob./ E. coli, Citrobacter, Salmonella, Serratia</i>
10/2008	<i>Klebsiella</i>	<i>Aeromonas/Enterob./ E. coli, Salmonella enteritidis</i>	<i>Aeromonas/Enterob./ E. coli, Klebsiella, Salmonella enteritidis</i>
11/2008 <sup>‡</sup>	<i>Hafnia, Klebsiella, Providencia/Pseudom./ Shigella, Serratia</i>	<i>Aeromonas/Enterob./ E. coli, Hafnia, Serratia, Salmonella</i>	<i>Aeromonas/Enterob./ E. coli, Salmonella</i>
12/2008	<i>Aeromonas/Enterob./ E. coli, Klebsiella, Salmonella enteritidis</i>	<i>Aeromonas/Enterob./ E. coli, Klebsiella</i>	<i>Aeromonas/Enterob./ E. coli, Klebsiella, Salmonella</i>
01/2009	<i>Aeromonas/Enterob./ E. coli, Klebsiella, Salmonella enteritidis</i>	<i>Aeromonas/Enterob./ E. coli, Klebsiella, Serratia</i>	<i>Aeromonas/Enterob./ E. coli, Salmonella enteritidis</i>
02/2009	ND	ND	ND
03/2009	<i>Aeromonas/Enterob./ E. coli, Citrobacter</i>	<i>Aeromonas/Enterob./ E. coli, Serratia</i>	<i>Aeromonas/Enterob./ E. coli</i>

ND - none detected

<sup>‡</sup>Sampling after rainfall

Salmonellae were detected in 30.8% (for RV broth) and 46.2% (for SC broth) of water samples from Berg 1, but a combined presence in 69.2% of samples (for RV and SC broth). *S. enteritidis* was frequently identified from both enrichment media. This organism is frequently implicated in salmonellosis which manifests in the gastrointestinal tract, as well as extra-intestinal infections which can affect the cardiac and pulmonary systems (Cohen *et al.*, 1987). In addition, a wide variety of genera which are members of *Enterobacteriaceae*, as well as isolates belonging to the genera *Aeromonas* and *Pseudomonas*, were isolated from the water. The latter genus encompasses the emerging opportunistic pathogen *P. aeruginosa*, other species which are associated with nosocomial infections as well as *P. putida*, which is capable of growth at 4°C (Fonseca *et al.*, 2011), the refrigeration temperature of many MPFs. *Aeromonas* spp. are autochthonous bacteria in fresh water, but *A. hydrophila*, *A. caviae* and *A. veronii* are pathogenic and can cause gastroenteritis and extraintestinal infectious disease in humans (Vila *et al.*, 2002). The disease-causing potential of these organisms found in water of the upper Berg River should be



a cause for concern to producers using the water for the irrigation of edible crops as well as persons using it for domestic and recreational use.

*Salmonella* spp. were detected in 7.7% (for RV broth) and 38.5% (for SC broth) of water samples obtained from Berg 2, with a combined presence in 38.5% of samples from this site (for RV and SC). This is slightly lower than the incidences found in water from Berg 1. Despite the lower incidence of detection, *Salmonella enteritidis*, *Aeromonas* and various genera of the *Enterobacteriaceae* family were frequently detected, and pose the same health risks as discussed for Berg 1 either through direct contact or indirect exposure through the consumption of river-irrigated produce.

The water samples sourced from Berg 3 contained *Salmonella* spp. in 61.5% (for RV broth) and 76.9% (for SC broth) of cases. The combined RV and SC results showed a presence of *Salmonella* in 84.6% of samples from this site. These frequencies are higher than that of Berg 1 and Berg 2, and corroborate the theory that pollution increases as the river progresses further downstream.

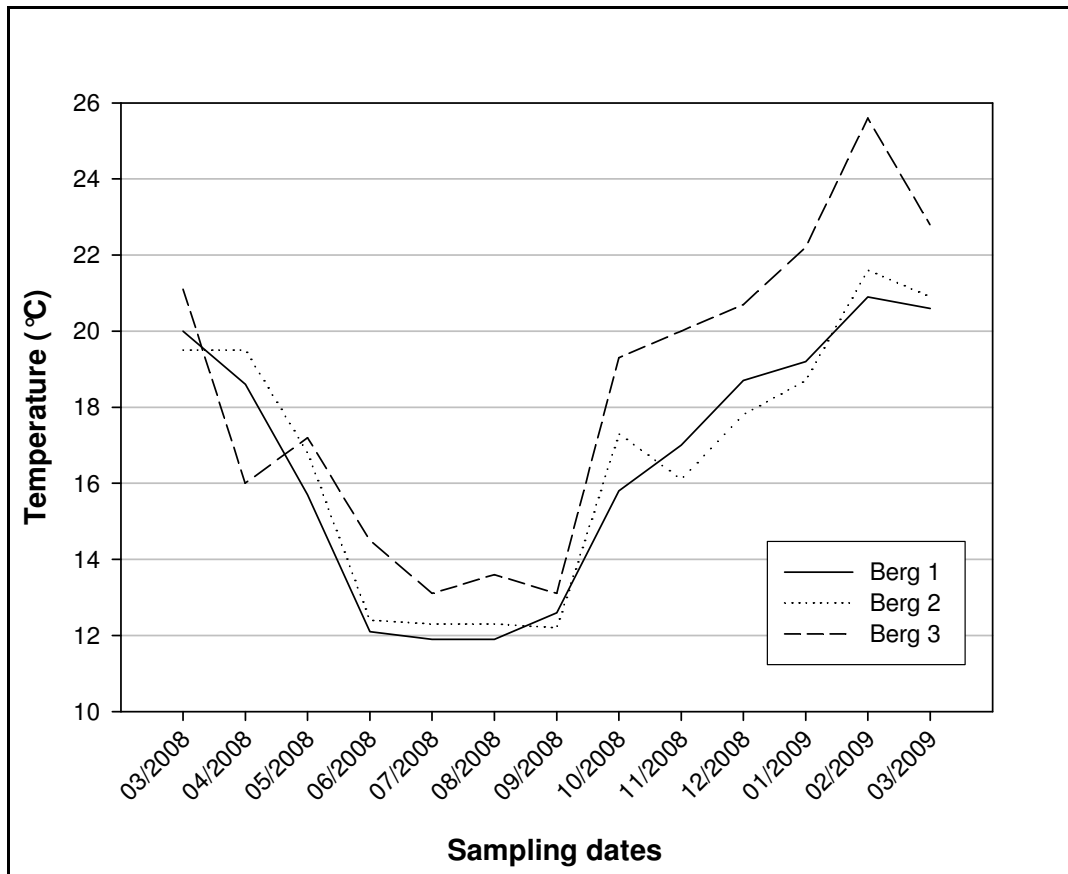
#### Coagulase-positive staphylococci

Coagulase-positive staphylococci were not detected in water from Berg 1 and 2, and only detected once in water from Berg 3 (10/2008, 470 cfu.mL<sup>-1</sup>). Since ACC were found for all of the sites in most cases, the lack of detection of coagulase-positive staphylococci is not due to an absence of bacteria in the water. The most plausible explanation is that coagulase-positive staphylococci are not typically found in faecal pollution, which has been shown to be the likely source of contamination of the water sampled at these sites. These bacteria are normally associated with the skin and upper respiratory tract of humans and animals (Harry, 1967; Wannamaker, 1970; Von Eiff *et al.*, 2001; Goodwin & Pobuda, 2009) which could account for their non-detection in the water of the upper Berg River. Alternatively, it is possible that the non-detection is attributable to the medium, and that low numbers of environmental coagulase-positive staphylococci could not be cultured on Baird-Parker agar with egg yolk tellurite emulsion. Patterson (1973) compared the ability of an agar medium similar to Baird-Parker with that of an MPN method to detect low levels of *Staphylococcus aureus* in foods. This study found that the MPN method was more effective in detecting *Staph. aureus* (Patterson, 1973). In addition, Goodwin and Pobuda (2009) evaluated the performance of CHROMagar *Staph aureus* and CHROMagar MRSA for the detection of environmental *Staph. aureus* and methicillin resistant *Staph. aureus* (MRSA) from beach sand and seawater. These workers found that detection was improved through the use of additional colony selection criteria (matte halo) as well as an amendment to the incubation time of the media (Goodwin & Pobuda, 2009), confirming that environmental staphylococci may require amended detection methodology.

### 3.4.2. Physical and chemical parameters

#### Temperature

The temperatures of the river water at the three samplings sites are given in Fig. 3.4. These results show that the water temperatures for the three sites were the lowest during 07/2008, 08/2008 and 09/2008. The former two correspond with winter months in South Africa and while September marks the official start of spring, day temperatures can still be low in the morning, when sampling was conducted



**Figure 3.4** River water temperature variations over 13 months for three sites

The expected decrease in temperatures from autumn to winter months, as well as an increase in temperatures from winter to spring and summer months can be observed. The increase in mean temperature with progression downstream is most probably attributable to the sampling procedure, which saw sampling occurring in numerical sequence (i.e. from Berg 1 to Berg 2 to Berg 3) while the day temperature was rapidly increasing during the course of the morning. This is confirmed by the temperature result for Berg 3 in 04/2008, when the site was sampled individually and consequently earlier than usual, which is considerably lower than the preceding month for the site as well as Berg 1 and Berg 2 for 04/2008.

### Alkalinity, conductivity and pH

The results of the alkalinity, conductivity and pH determinations for river water samples from three sites are given in Table 3.9 on the next page. The alkalinity values in the table show few trends in terms of season, downstream progression or identified pollution spikes. The only prominent feature of the results is the increase in alkalinity for all three sites in 06/2008. This date has been identified during the discussion of the microbiological analyses as being the start of heavy winter rainfall, which consequently causes flushing of pollution on the riverbank into the river system. If these events are related, the pollution could possibly have contributed to the high alkalinity values.

**Table 3.9** Alkalinity, conductivity and pH values for river water samples from three sites<sup>†</sup>

Sampling date	Berg 1			Berg 2			Berg 3		
	Alkalinity	Conductivity	pH	Alkalinity	Conductivity	pH	Alkalinity	Conductivity	pH
03/2008	12.5	2.93	5.49	12.5	8.39	5.55	12.5	6.76	5.61
04/2008	5.0	4.07	5.31	12.5	6.84	5.39	50.0	27.50	5.72
05/2008	5.0	0.06	5.45	12.5	0.05	5.56	25.0	0.13	5.69
06/2008	625.0	6.55	5.26	125.0	7.84	5.29	125.0	11.96	5.26
07/2008 <sup>‡</sup>	25.0	8.47	6.52	12.5	9.14	6.24	12.5	12.94	6.13
08/2008	12.5	7.30	6.39	12.5	9.35	6.62	25.0	14.18	6.73
09/2008	12.5	6.06	6.48	12.5	5.27	6.38	12.5	10.13	6.38
10/2008	12.5	8.38	7.03	12.5	9.47	6.95	37.5	16.02	6.94
11/2008 <sup>‡</sup>	12.5	4.57	6.03	12.5	4.54	6.07	12.5	7.41	6.20
12/2008	12.5	4.64	5.92	12.5	5.02	6.08	12.5	9.11	6.09
01/2009	12.5	2.78	5.30	12.5	3.14	5.31	12.5	7.50	5.79
02/2009	12.5	2.74	4.50	12.5	3.14	4.75	12.5	6.65	5.82
03/2009	<2.0	2.25	4.30	12.5	2.72	5.00	12.5	6.74	5.10

<sup>†</sup>Alkalinity as mg CaCO<sub>3</sub>.L<sup>-1</sup>, conductivity as mS.m<sup>-1</sup> and pH (-log<sub>10</sub> [H<sup>+</sup>])

<sup>‡</sup>Sampling after rainfall

As it can be seen from the results reported in Table 3.8, conductivity readings showed a slight increase as sites progressed downstream. This measurement is used to determine the amount of total dissolved salts in water, and their ability to facilitate the conduction of electrical charge. It is very likely that these measured ions are present in the faecal pollutant, and that their elevated presence in the river water increased the conductivity values. The South African guideline of the acceptable range for conductivity of water used for irrigation is 0-40 mS.m<sup>-1</sup> (DWAf, 1996). When the results in Table 3.9 are compared with this national guideline it is clear that the conductivity of water samples was within the limits in all of cases.

From the results in Table 3.9 it was found that the pH of water at all sites was low in March of 2008, and steadily showed an increase until 10/2008. After this date, the pH values of the water at all sites started decreasing and were very low in March of 2009. The pH of Berg 1 on this date, at 4.30, was exceptionally low. This value coincided with an alkalinity value of zero for the water

obtained from this site in 03/2009. These observations were made during a time of year when viticultural activities located on the riverbank are increased due to the harvesting and pressing of grapes, and effluent produced by these industries could, in some part, be responsible for the observed decrease in pH.

The acceptable pH range for irrigation water, according to the FAO and DWAF, is between 6.5 and 8.4 (DWAF, 1996). Water with a pH outside these limits are considered to be of unacceptable quality (FAO, 2003) and, consequently, inappropriate for irrigation. If these regulations, which are accepted by the United Nations, are applied to the results in Table 3.9, the incidences of transgression would be 84.6% for all sites. One incident of compliance occurred on the same date (10/2008) for all sites, at a time when winery and distillery activities are limited.

#### Chemical oxygen demand (COD)

The results of the COD analyses for river water samples from three sites are given in Table 3.10.

**Table 3.10** COD values for river water samples from three sites

Sampling date	Chemical oxygen demand (as mg O <sub>2</sub> .L <sup>-1</sup> )		
	Berg 1	Berg 2	Berg 3
03/2008	9.0	15.3	42.3
04/2008	81.0	1.8	3.6
05/2008	21.6	12.6	36.9
06/2008	11.7	22.5	8.1
07/2008 <sup>‡</sup>	17.1	9.0	66.6
08/2008	41.4	36.9	28.8
09/2008	14.4	<3.0	81.0
10/2008	1.8	<3.0	9.0
11/2008 <sup>‡</sup>	<3.0	<3.0	<3.0
12/2008	<3.0	<3.0	<3.0
01/2009	18.9	39.6	36.9
02/2009	24.3	15.3	13.5
03/2009	12.6	21.6	9.0

<sup>‡</sup>Sampling after rainfall

The COD limit for effluent discharged into listed rivers, such as the Berg River, is 30 mg O<sub>2</sub>.L<sup>-1</sup> in South Africa (DWAF, 1999). It is clear from the results that Berg 1 and Berg 2 did not comply in 15.3% of cases, while Berg 3 transgressed this limit in 38.5% of cases. It is speculation to suggest that industries are responsible for the elevated COD levels found in the water of the upper Berg River, since the nature of the chemical contamination was not further examined. However, the discharge of chemical effluents should be more closely and regularly policed by

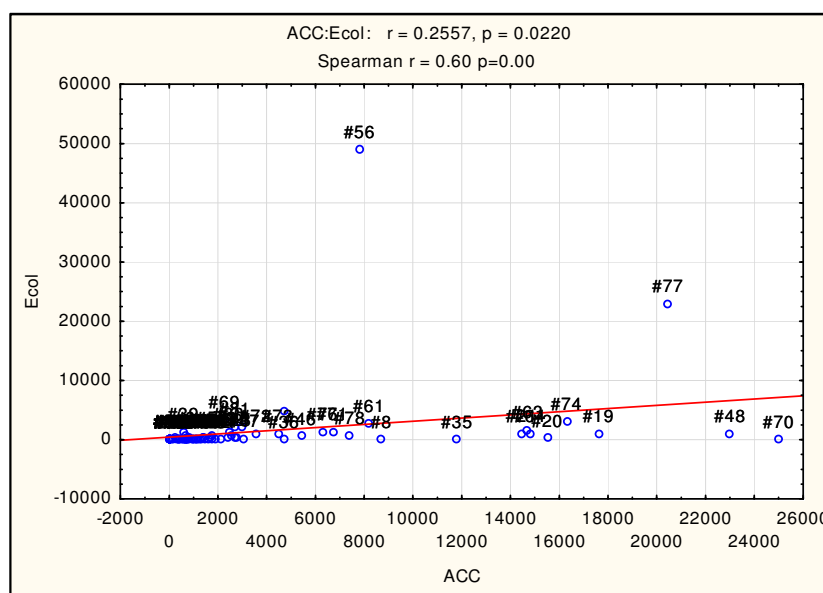
governmental bodies to ensure that the limits are strictly adhered to, to prevent a decline in the quality of the water of the upper Berg River.

### 3.4.3. Statistical correlation of quantified parameters with *E. coli* concentration

Spearman correlations - ability of individual parameters to predict *E. coli* numbers

#### *Microbiological parameters*

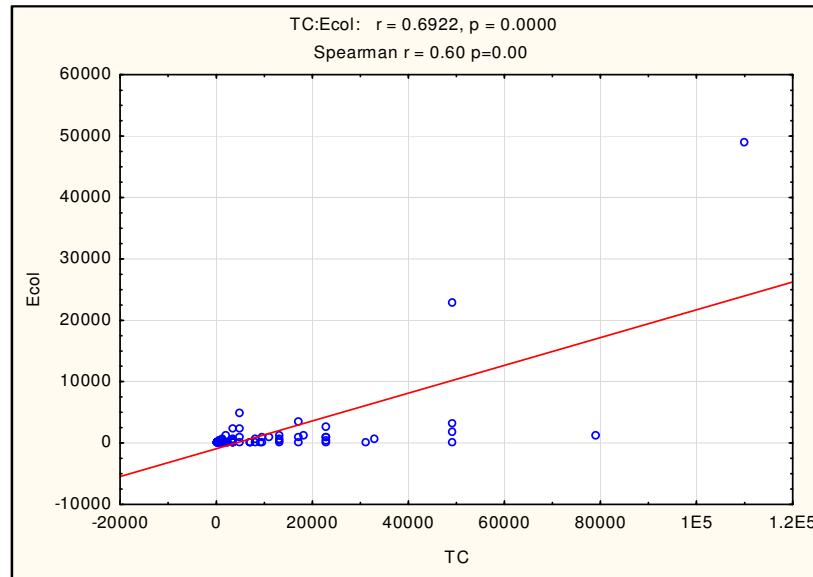
The Spearman correlation between ACC and *E. coli*, as well as the Spearman rank correlation coefficient, is depicted in Fig. 3.5.



**Figure 3.5** Spearman correlation between aerobic colony count (ACC) and *E. coli* (Ecol)

As indicated in the figure, the Spearman rank correlation coefficient for ACC and *E. coli* is  $r=0.60$ . Therefore, the  $r^2$  value is 0.36, which indicates that aerobic colony counts are extremely poor predictors of *E. coli* numbers and can only account for 36% of variation in *E. coli* numbers in water from the upper Berg River.

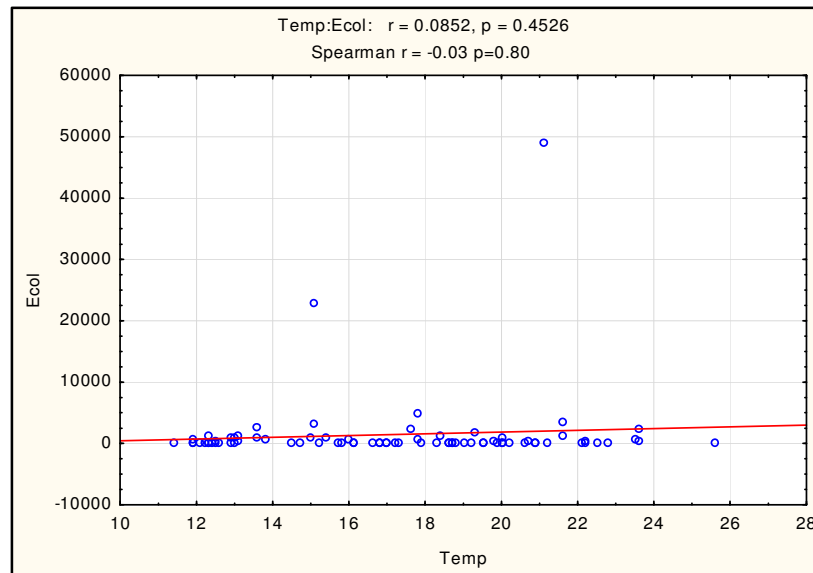
The Spearman correlation between coliforms and *E. coli*, as well as the Spearman rank correlation coefficient for this correlation, is depicted in Fig. 3.6 (shown on the next page). The Spearman rank correlation coefficient for coliform and *E. coli* numbers is identical to that obtained for ACC. The  $r^2$  value for this correlation is 0.36, which is indicative of a very poor predictor of *E. coli* numbers. This is surprising, since coliforms are a considerably more defined group when compared with aerobic bacteria. These results indicate, however, that the coliforms, a group which includes *E. coli*, are no better than ACC for the prediction of *E. coli* numbers.



**Figure 3.6** Spearman correlation between coliforms (TC) and *E. coli* (Ecol)

#### *Physical and chemical parameters*

The Spearman correlation between water temperature, a physical parameter, and *E. coli* is depicted in Fig. 3.7. The Spearman rank correlation coefficient is also shown in the figure.

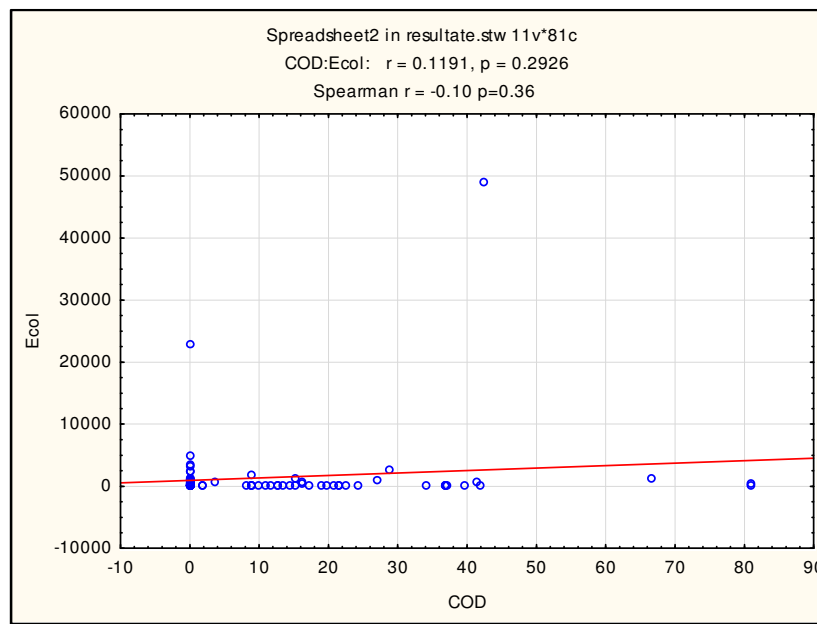


**Figure 3.7** Spearman correlation between temperature (Temp) and *E. coli* (Ecol)

The correlation between water temperature values and *E. coli* numbers was extremely weak. The Spearman rank correlation coefficient was very low at -0.03, or an  $r^2$  value of 0.0009.

In addition, the two parameters were inversely correlated. These results indicate that water temperature is an entirely inappropriate parameter for the prediction of *E. coli* numbers for river water from the upper Berg River.

The Spearman correlation between the first chemical parameter, COD, and *E. coli* is depicted in Fig. 3.8. The Spearman rank correlation coefficient is also shown in the figure.

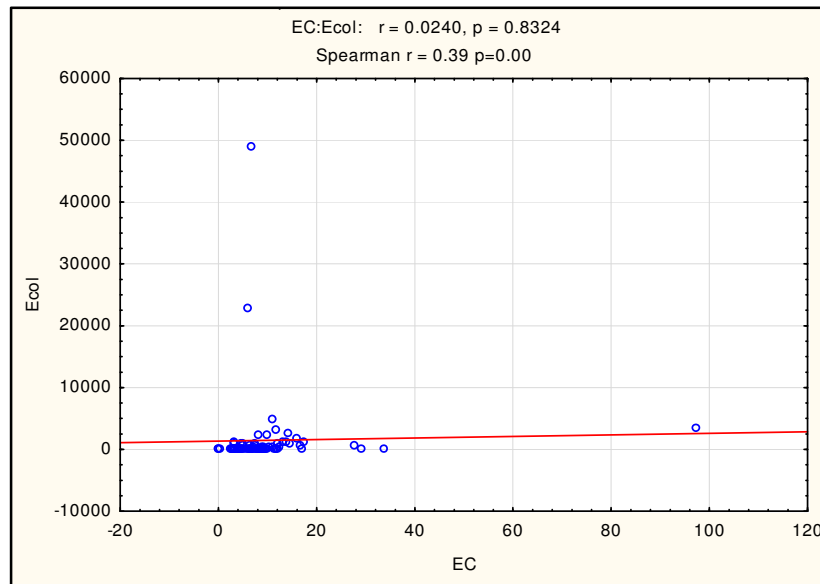


**Figure 3.8** Spearman correlation between COD and *E. coli* (Ecol)

The correlation of the COD parameter with *E. coli* was, similar to that of temperature, very weak and inversely correlated. This inversion, in other words, indicate that as COD values tend to rise *E. coli* numbers tend to decrease. The Spearman rank correlation coefficient is -0.1, and the corresponding  $r^2$  value is 0.01. This result confirms that the use of COD values as replacement of *E. coli* numbers, or faecal pollution, is entirely inappropriate and that it cannot be used as an indicator of faecal pollution in the upper Berg River.

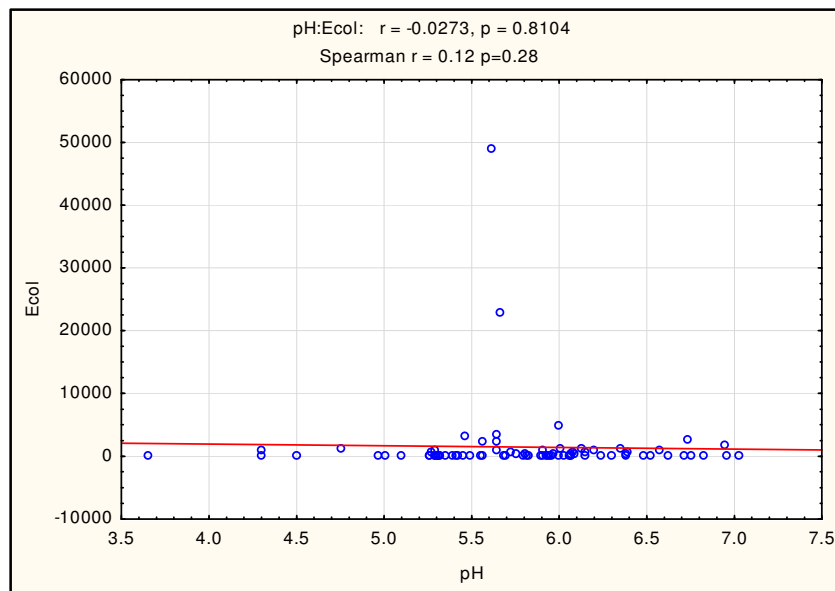
The Spearman correlation between conductivity and *E. coli* is depicted in Fig. 3.9 on the next page. The Spearman rank correlation coefficient indicated in Fig. 3.8 indicates that this chemical parameter performed better than temperature and COD in the prediction of *E. coli*. Despite the direct correlation between the two variables, the correlation coefficient was still very low, with an  $r^2$  value of 0.15. This value indicates that variation in conductivity could only account for 15% of variation in *E. coli*. Therefore, this parameter is also inappropriate for accurate prediction of *E. coli* numbers in river water from the upper Berg River.





**Figure 3.9** Spearman correlation between conductivity (EC) and *E. coli* (Ecol)

The Spearman correlation between pH and *E. coli* is depicted in Fig. 3.10. The Spearman rank correlation coefficient is also shown in the figure.



**Figure 3.10** Spearman correlation between pH and *E. coli* (Ecol)

The correlation in Fig. 3.10 clearly show that the pH of the river water has an extremely weak correlation with *E. coli* numbers, as was the case with all other chemical parameters. The correlation between pH and *E. coli* is a direct correlation, but the low correlation coefficient and  $r^2$  value (0.0144) indicate that pH is not an appropriate predictor of *E. coli* numbers in water from the upper Berg River.

### Regression analysis - predictive ability of combined parameters

The regression analysis was done to determine the extent to which the combination of the six parameters namely ACC, coliforms, water temperature, COD, electrical conductivity and pH can predict the *E. coli* numbers in river water from the upper Berg River. The correlation coefficient, with exclusion of two outliers, was calculated as 0.704. Consequently, the Multiple  $R^2$  and Adjusted Multiple  $R^2$  values were 0.496 and 0.446, respectively. These results show that the combination of measured parameters accounted for less than half (44.6%,  $p < 0.01$ ) of the variation in *E. coli* numbers. Therefore, the remaining 55.4% of variation in *E. coli* is caused by unknown parameters which were not measured in this study.

### Durbin-Watson d and serial correlation - combined parameters

The Durbin-Watson d serial correlation analysis obtained a serial correlation of 0.32 and corresponding  $r^2$  value of 0.10. The Durbin-Watson statistic was calculated as 1.25. This statistical analysis is used in the determination of autocorrelation in the prediction errors of the regression analysis. Since the Durbin-Watson statistic is less than 2, the serial correlation is positive and positive prediction errors in one measurement will increase the likelihood of positive prediction errors for another measurement. Therefore, the unknown factors causing an increase in one measurement, e.g. ACC, is likely to lead to an increase in another measurement, e.g. *E. coli*.

## 3.5. CONCLUSIONS

### 3.5.1. State of the upper Berg River

The results give clear indications that the upper Berg River is not microbiologically pristine. The microbiological contamination has been shown to most probably be faecal in origin, due to the increases in *E. coli* from the upstream baseline site to the downstream baseline site. Further supporting evidence to this effect are the increases in ACC and coliform counts with progression downstream. However, the results shown here indicate that the upper Berg River is not as heavily polluted as was previously believed, and is currently under less pollution pressure than other rivers, such as the Plankenburg, which were examined in the area (Kikine, 2011).

In addition to high levels of the indicators of general microbiological quality and specific faecal contamination, aggressive as well as opportunistic human pathogens were also detected in water from all sites. Of particular concern in this regard was the high frequency of *Salmonella* spp. detection in samples from all sites. These pathogenic bacteria pose various health risks to individuals living in close proximity to the river, those who use the water for domestic and recreational contact, as well as consumers who unwittingly purchase and consume minimally

processed foods irrigated with this water. These organisms are potential agents of large-scale outbreaks, which would not only directly damage the economy through medical subsidies, loss of income and a decreased workforce, but also through the reputation of South Africa as an exporter of minimally processed foods.

The chemical parameters which were measured for the three sites also raised some concerns: the COD values outside of the effluent discharge limit as well as the low pH of water samples should be a source of concern for both the irrigation of crops as well as the ecology of the river. Industrial effluent discharge in this area should be more effectively policed, and, if implicated, industries should be fined for partaking in such ecologically and, eventually, economically damaging practises.

### **3.5.2. Microbiological methods**

The results from this study showed that the specific detection of *L. monocytogenes* is more effective during the secondary enrichment step in full Fraser broth. The primary enrichment step, in contrast, was more proficient at detecting other listerial species.

The results for the detection of *Salmonella* spp. indicate that as a secondary enrichment step, SC broth was far more sensitive in the detection of *Salmonella* spp. and *S. enteritidis* than was RV broth. Both media were found to support the growth of a variety of other enteric and non-enteric genera. It does appear from these results that the SC broth is more appropriate if *Salmonella* spp. needs to be specifically detected.

### **3.5.3. Predictive value of non-*E. coli* parameters for the prediction of *E. coli* numbers**

The Spearman rank correlation statistics prove that the use of ACC, coliform number, water temperature, COD, electrical conductivity and pH as replacements for *E. coli* numbers are inappropriate and would result in serious inaccuracies of estimation. These results prove that for water from the upper Berg River none of these parameters can be used as predictors of *E. coli* numbers. COD, in particular, is frequently used in industries such as wastewater treatment works to “determine” the degree to which faecal pollution has been removed. The work presented here is strongly discouraging of such an approach.

The results of the regression analysis showed that the measured parameters which were used during this study could not even account for half of the variation in *E. coli* numbers. This finding indicates that many unknown parameters which could influence *E. coli* numbers were not included in the study. Any identification of such unknown factors would amount to speculation, but the utilisation of ambient day or night temperature rather than water temperature may have slightly increased the predictive ability since water is rather inert to air temperature fluctuations.

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## CHAPTER 4

### EVALUATION OF COLILERT-18 AS ALTERNATIVE METHOD FOR MULTIPLE TUBE FERMENTATION IN THE ENUMERATION OF COLIFORMS AND *E. COLI* FROM SOUTH AFRICAN RIVER WATER

#### 4.1. ABSTRACT

The Colilert-18 method was evaluated as a rapid alternative for multiple tube fermentation (MTF) in the enumeration of coliforms and *E. coli*. Samples from four rivers in the Western Cape of South Africa were used in the comparative study which used both methods to enumerate coliforms and *E. coli* from the water. The rapid enumeration of these organisms from river water holds promise for faster preventative or corrective action in the event of irrigation of fresh produce with water of an unacceptable microbiological quality. The MTF results indicated instances where *E. coli* levels exceeded the World Health Organization (WHO) guideline for irrigation water for the Berg (32.3% of samples), Plankenburg (95.0% of samples), Eerste (22.2% of samples) and Lourens (100.0% of samples) Rivers. The comparative results and agreement frequency tables indicated that Colilert-18 showed low to intermediate agreement with MTF for coliforms and *E. coli*. In comparison with MTF, Colilert tended towards higher enumerations of coliforms in water from all rivers, presumably due to a higher coliform recovery rate. The Spearman rank correlation coefficient indicated a fair correlation ( $r^2=0.69$ ) between the two methods for the enumeration of coliforms. However, a Bland and Altman plot indicated that while Colilert agreed well with MTF in the range of 0 to 100 000 coliforms MPN.100 mL<sup>-1</sup>, Colilert showed an increasing tendency for higher enumerations above this limit. Colilert produced lower enumerations for *E. coli*, with the exception of samples from the upper Berg River. The Spearman rank correlation coefficient showed a fair correlation ( $r^2=0.74$ ) between the two methods for the enumeration of *E. coli*, but a Bland and Altman plot indicated that as *E. coli* counts exceeded 50 000 MPN.100 mL<sup>-1</sup> Colilert showed an increasing tendency towards lower *E. coli* enumerations. Colilert is subsequently considered to be an acceptable rapid alternative for the enumeration of coliforms and *E. coli* from South African river water when these counts do not exceed 100 000 coliforms MPN.100 mL<sup>-1</sup> and 50 000 *E. coli* MPN.100 mL<sup>-1</sup>. Considering the state of the four rivers examined in the study, the improved response time resulting from this rapid method could result in faster reaction times to occurrences of irrigation of fresh produce with faecally contaminated river water.

## 4.2. INTRODUCTION

The ability of the MTF method to accurately enumerate coliforms, thermotolerant coliforms and *E. coli* using most probable number (MPN) statistical tables (Christensen *et al.*, 2002) has seen it become the method of choice for the analysis of faecal contamination of samples. It is also the method which is typically used when water quality determinations are done in developing countries (Macy *et al.*, 2005). The accuracy of the method is attributable to various selective hurdles which ensure that the intended group of test organisms alone are detected.

There are several practical and scientific drawbacks to the method. A large number of coliforms, and 5% of *E. coli* (Moberg, 1985) do not produce gas during lactose fermentation (Fricker *et al.*, 1997), the principle used to detect these organisms with MTF. In addition, the method is expensive due to the utilisation of three broth steps and a fourth confirmatory step on Levine-eosin methylene blue (L-EMB) agar (Christensen *et al.*, 2002), highly labour intensive, and results are only available after two (for preliminary results) (WHO, 2001) to four days (for final results) (Moberg, 1985; Geissler *et al.*, 2000; Maheux *et al.*, 2008). Such time lapses cause a considerable delay before corrective action can be taken. This has resulted in a need for more rapid enumeration methods for water and, especially, in the event of an emergency (IWA, 2000).

The defined substrate technology Colilert method has only recently gained popularity for the rapid enumeration of coliforms and *E. coli*, after automation was improved and the Quanti-Tray for enumeration was developed. This method has several practical advantages over MTF, as analysis is more automated and results are available within 18-22 hours (Fricker *et al.*, 2008b; Wohlsen *et al.*, 2008). Furthermore, anaerogenic coliforms (Fricker *et al.*, 1997) do not affect the results (Wohlsen *et al.*, 2008) as is the case with MTF, which relies on the production of gas through the fermentation of lactose.

The development of the Quanti-Tray enabled Colilert to be compared with other *E. coli* enumeration methods, and extensive research has been done worldwide to compare Colilert with data from membrane faecal coliform (mFC) agar (Wohlsen *et al.*, 2008); MTF (Eckner, 1998; Kämpfer *et al.*, 2008); membrane filtration on membrane lauryl sulphate broth (MLSB) (Fricker *et al.*, 1997), Tergitol 2,3,5-triphenyltetrazolium chloride (TTC) agar (Niemela *et al.*, 2003; Fricker *et al.*, 2008a), lactose TTC agar (Kämpfer *et al.*, 2008) and membrane lactose glucuronide agar (MLGA) (Fricker *et al.*, 2010). These comparisons were done using a variety of test samples including bacterial culture solutions, drinking water, chlorinated water, groundwater, surface water and sewage effluent. All researchers concluded that Colilert was a viable alternative exhibiting similar, or even better, performance.

Despite these positive results, it is clear from the differences in test samples used in the above findings that Colilert should be tested for a specific type of sample against the traditional method that it is aiming to replace, before such a replacement is made. In addition, Covert *et al.* (1989) stated that the test should not be applied to any water sample “unless the analyst

establishes the efficacy of the AC (Autoanalysis Colilert, the first developmental phase of Colilert) test with the particular sample type”.

It is known that the composition of river waters located in the same area can vary greatly (Livingstone, 1963; Chételat *et al.*, 1999). These differences in composition are a function of the type of activities occurring in proximity to the river environment; the number of humans using the river, consciously or inadvertently, for domestic purposes including sanitation; as well as the size and flow of the river. Therefore, the evaluation of the suitability of Colilert as an alternative method to MTF for the monitoring of the rivers cannot be done using water from one river system. The South African Department of Water Affairs and Forestry (DWAF) monitors rivers throughout the country through the National Microbial Monitoring Programme (NMMP), using membrane filtration or MPN (MTF) to determine the faecal (thermotolerant) coliform count (DWAF, 2002). In addition, the baseline monitoring of river water has been done for more than three years for the Water Research Commission (WRC) solicited research project K5/1773, of which this study forms a part. However, no comparative studies have been done on the efficacy of Colilert as an alternative to MTF, despite the potential benefit of more rapid results which can aid in the early warning of vulnerable communities and producers of fresh produce which, in turn, can prevent the occurrence of water- and foodborne outbreaks.

The aims of this study were: (1) to determine whether Colilert is an appropriate rapid alternative for MTF in the analysis of selected South African rivers; and (2) to determine whether there is a difference in the performance of Colilert at high, intermediate and low levels of faecal pollution.

### **4.3. MATERIALS AND METHODS**

#### **4.3.1. Site selection**

Water samples were drawn from four rivers in the Western Cape: the Plankenburg, upper Berg, Eerste and Lourens Rivers. The two sites in the Plankenburg River were designated Plank 1 and 3. Plank 1 is situated approximately 1 km downstream from the Kayamandi large informal settlement. Plank 3 occurs at the confluence of the Plankenburg with the Eerste River, *ca.* 2 km downstream from Plank 1, after the river has flowed past various industrial sites and informal dwellings on the riverbank. For the purpose of the study this river was categorised as “more polluted”.

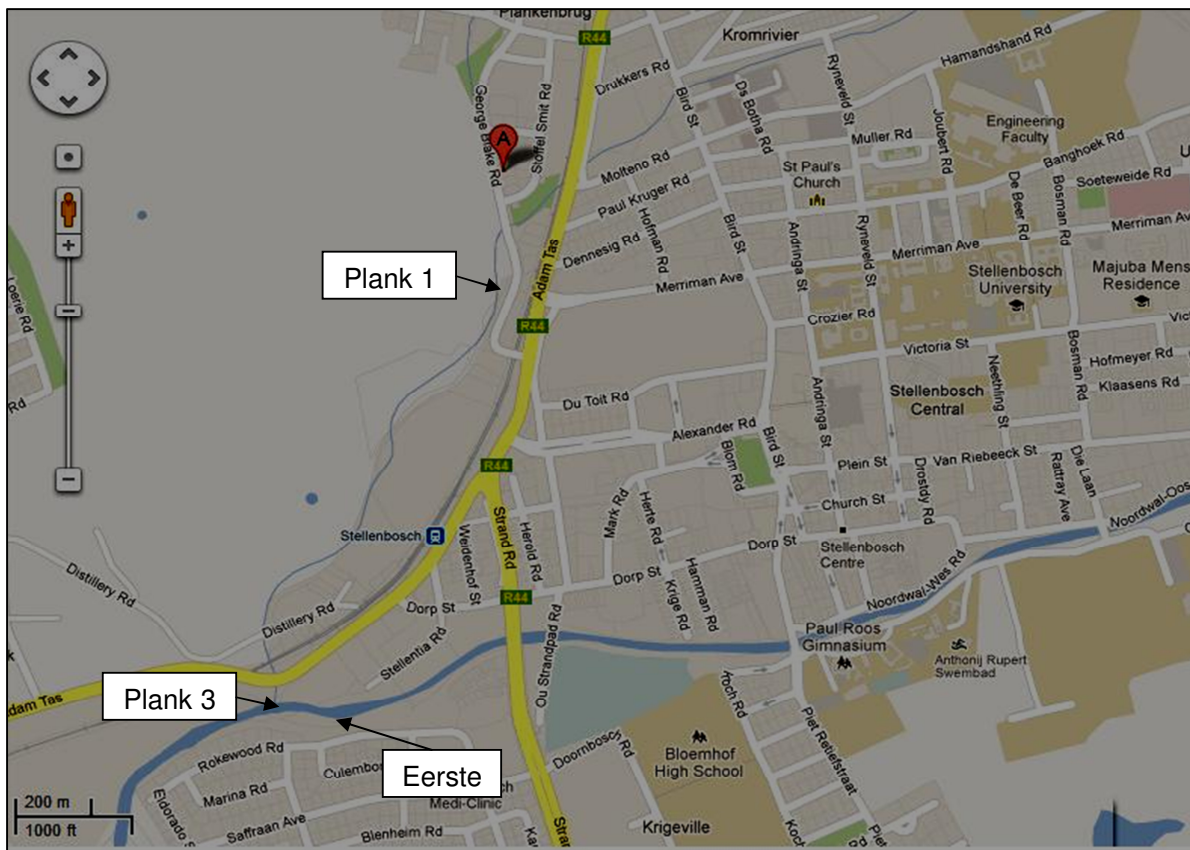
Three sampling sites in the upper Berg River were selected, as water from this river is used as the main source of irrigation for the production of vegetables, export grapes and deciduous fruit in addition to its application for various other agricultural activities (DWAF, 2004). These sampling sites were the same three sites which were analysed in Chapter 3. Briefly, site Berg 1 is situated approximately 7 km downstream from the Berg River Dam and the confluence of the Franschhoek/Stiebeuel Rivers with the Berg River. The site Berg 2 occurs approximately 20 km

downstream from Berg 1, and is situated downstream from a commercial farm producing tomatoes for sale export and local markets. The third site, Berg 3, is 30 km downstream from Berg 2, and is located 500 m downstream from the Lady Loch Bridge on the outskirts of Wellington. The Berg River is a much larger river system than the Plankenburg and carries a much larger volume of water, which in turn results in a more pronounced dilution effect on contaminants. The water in some stretches of the river is still fairly clean, and was categorised for the purpose of the study as “less polluted”.

The sampling site in the Eerste River is located just before the river converges with the Plankenburg River. This river is considered to be “less polluted” than the Plankenburg River.

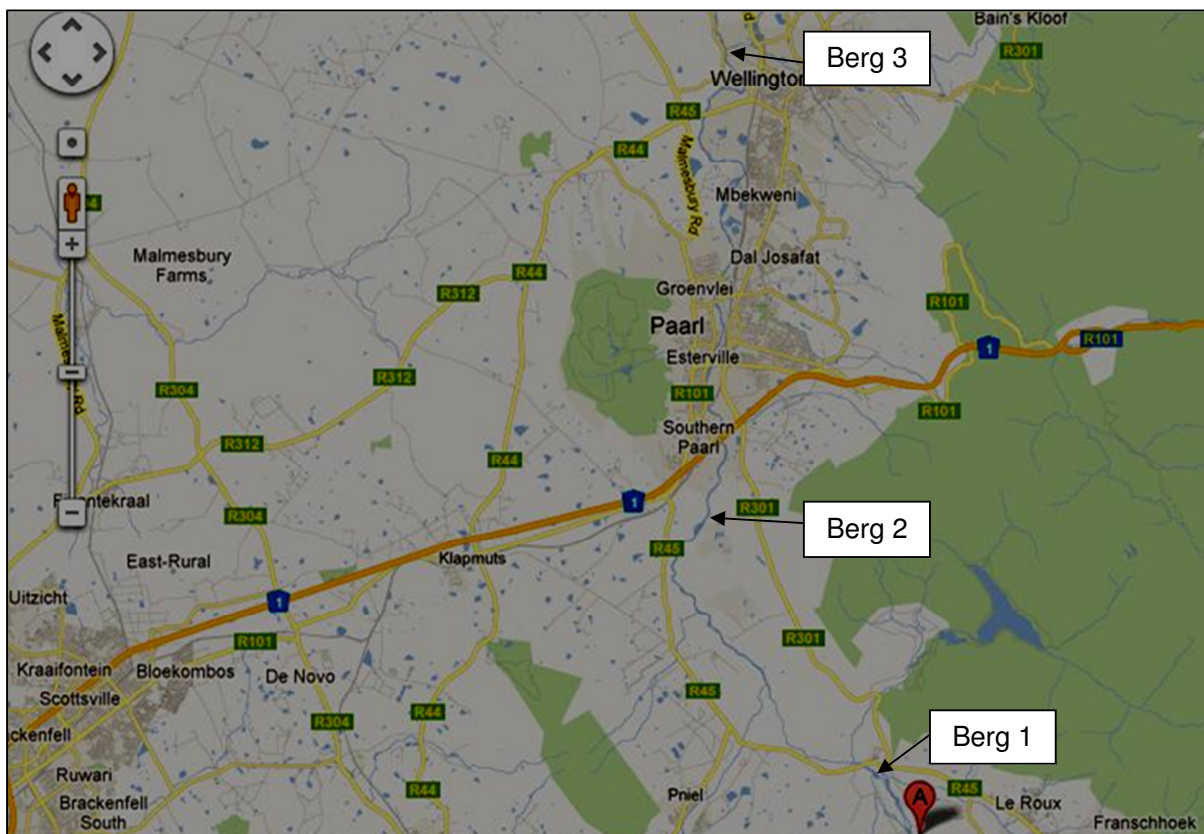
The Lourens River has its origin in the Helderberg Mountains. The river flows through the towns of Somerset West and Strand before draining into False Bay. The site selected is situated 1 kilometre before the river flows into the ocean. The Lourens River is less polluted than the Plankenburg, but carries a fair amount of urban runoff and pollution and is classified for this study as “more polluted”.

The geographical locations of sites in the Plankenburg and Eerste Rivers are, due to their close proximity, indicated together in Figure 4.1. The locations of sites in the upper Berg and Lourens Rivers are shown in Figures 4.2 and 4.3, respectively.



**Figure 4.1** Geographical locations of sampling sites in the Plankenburg and Eerste Rivers (Map data ©2012 AfriGIS (Pty) Ltd, Google)





**Figure 4.2** Geographical locations of sampling sites in the upper Berg River (Map data ©2012 AfriGIS (Pty) Ltd, Google)



**Figure 4.3** Geographical location of sampling site in the Lourens River (Map data ©2012 AfriGIS (Pty) Ltd, Google)

#### 4.3.2. Sample collection

For each site, 1 L of river water was drawn in a sterile glass container using the guidelines according to Standard Methods for the Examination of Water and Wastewater (2005). Samples were placed on ice and analysed within four hours. Sampling was done on a monthly basis from two sites along the Plankenburg River for ten consecutive months, from three sites along the upper Berg River in May 2009 and for ten consecutive months from July 2009, from one site in the Eerste River for ten consecutive months and twice at one site in the Lourens River.

#### 4.3.3. Microbiological analyses

Water samples were analysed for coliforms and *E. coli* using the MTF method (MFHPB-19) as described by Health Canada (Christensen *et al.*, 2002). Lauryl sulphate tryptose (LST) and brilliant green lactose bile (BGLB) broths (Oxoid, Basingstoke, Hampshire, UK) showing growth and gas production were used for the detection of presumptive and confirmed coliforms. *E. coli* (EC) broth (Oxoid, Basingstoke, Hampshire, UK) with 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG) (Oxoid, Basingstoke, Hampshire, UK) showing growth, gas production and fluorescence, as well as confirmatory L-EMB agar (Oxoid, Basingstoke, Hampshire, UK) plates showing typical dark colonies with metallic green sheen, were used for the detection of *E. coli*. The enumeration of these organisms was done using the De Mans MPN table (Standard Methods for the Examination of Water and Wastewater, 1995; Christensen *et al.*, 2002) and results were reported as coliforms and *E. coli* MPN.100 mL<sup>-1</sup>.

Aliquots from the same samples were also analysed by the Colilert method using the protocol described by the manufacturer (IDEXX Laboratories, 2011), Colilert-18 reagent and Quanti-Tray 2000. Samples were diluted tenfold to an appropriate dilution, using 90 mL portioned sterile saline solution (0.85% w/v NaCl (Biolab, Merck, Wadeville, South Africa) in dH<sub>2</sub>O), typically ranging between 10<sup>-2</sup> and 10<sup>-4</sup>.

#### 4.3.4. Method comparison

The instances where samples were either insufficiently or excessively diluted, which resulted in the value falling above the upper or below the lower boundary of the table, both the Colilert value and the corresponding MTF value for the sample were removed from the data set. Frequency tables were constructed, both for the entire river data sets and separate rivers, to determine the extent of agreement between Colilert and MTF in the enumeration of coliforms and *E. coli*.

The Spearman rank correlation coefficient (Estelberger & Reibnegger, 1995) between MTF and Colilert was calculated for both coliforms and *E. coli* for the entire four-river data set, due to the robustness of this correlation against outlier data (Bin Abdullah, 1990). Intra-class correlations (ICC) were also calculated for both coliforms and *E. coli*. The ICC (consistency) is similar to a

normal correlation, but the ICC (agreement) penalises the method which is biased to always measure higher. When a low ICC (agreement) and high ICC (consistency) is found, a correction factor can be calculated and the alternative method may substitute the old method with appropriate mathematical correction of the measurements (Prof. M. Kidd, Center for Statistical Consultation, Stellenbosch University, personal communication).

To determine the performance of Colilert when compared with MTF when analysing highly polluted, intermediately polluted and lightly polluted river water, Bland and Altman plots were constructed for both total coliforms and *E. coli* for the entire data set (Prof. M. Kidd, Center for Statistical Consultation, Stellenbosch University, personal communication).

#### 4.4. RESULTS AND DISCUSSION

##### 4.4.1. Comparison of methods

###### Coliforms

The data set for coliforms consisted of 56 MTF values and their corresponding Colilert values for each sample. When Colilert values could not be calculated due to excessive or insufficient dilution, the corresponding MTF value was also omitted from the data set. The distribution of the comparative coliform data pairs across rivers is given in Table 4.1.

**Table 4.1** Distribution of coliform data across four rivers

River	Proportion of comparative coliform data points (%)
Upper Berg	51.8
Plankenburg	26.8
Eerste	17.8
Lourens	3.6

The coliform data set for the Berg River is shown in Table 4.2. Mean, median and standard deviation values were calculated after exclusion of counts which fell outside the limits of the Colilert MPN table.



**Table 4.2** Coliform values (MPN.100 mL<sup>-1</sup>) for the Berg River sites, enumerated by Colilert and MTF

Sample date	Berg 1		Berg 2		Berg 3	
	Colilert	MTF	Colilert	MTF	Colilert	MTF
05/2009	*	*	3 199	350	19 863	9 200
07/2009	8 664	2 300	9 208	1 400	19 863	23 000
08/2009	5 794	2 300	11 199	23 000	*	*
09/2009	*	*	*	*	57 940	49 000
10/2009	17 200	7 000	7 244	790	54 750	18 000
11/2009	24 196	4 900	57 940	17 000	198 630	49 000
12/2009	51 720	3 300	15 531	490	8 664	7 900
01/2010	203	230	327	490	5 794	3 300
02/2010	24 196	1 300	19 863	580	6 867	1 300
03/2010	9 804	130	19 863	230	15 531	4 900
04/2010	3 578	130	8 664	460	19 863	3 300
<b>Mean</b>	16 151	2 399	15 304	4 479	40 777	16 890
<b>Median</b>	9 804	2 300	10 204	535	19 863	8 550

\* No data points were obtained for the specific site on this date

When the calculated means in Table 4.2 are compared for each site, it is clear that Colilert tends to produce considerably higher values than those produced by MTF for all sites in the upper Berg River. This phenomenon could possibly be attributed to the ability of Colilert to detect and enumerate anaerogenic coliforms (Fricker *et al.*, 1997; Wohlsen *et al.*, 2008), whereas MTF is unable to do so. For Berg 1, the least faecally polluted of the three sites in the upper Berg River (see Fig. 3.2), the mean Colilert value was nearly seven times higher than the mean value for MTF. For Berg 2, also lightly faecally polluted, this was slightly lower, with the mean for Colilert approximately 3.5 times higher than that for MTF. The mean Colilert value for water obtained from Berg 3, the most polluted, was only around 2.5 times higher than the mean value calculated for MTF. These observations are interesting, since it alludes to the possibility that Colilert enumerations for coliforms tend to agree more closely with MTF values in increasingly faecally polluted river water.

However, when the median values for the three sites are examined, it is clear that a larger difference was obtained between Colilert and MTF median values for Berg 2 than for Berg 1. The large difference observed in the mean values of the two methods for Berg 1 were attributable to isolated cases where Colilert enumerated considerably higher than MTF (in October, November and December 2009; February 2010), rather than a consistent production of slightly higher values by Colilert. In contrast, the large difference in median values, and especially the high Colilert median, for Berg 2 indicate that the difference in mean values were due to a consistent production

of considerably or severely higher enumerations by Colilert (except on two occasions), and not because of single considerably higher spikes in the data. “Considerably higher”, for the purpose of this discussion, was evaluated as values which were at least one order of magnitude greater than its corresponding MTF value. The difference which was observed for the median values of Berg 3 indicated that the difference in mean values were attributable to incidents or spikes of considerably higher enumeration by Colilert (May and November 2009; March and April 2010) rather than a consistent production of slightly higher values than those obtained by MTF.

These differences in median values indicate that Colilert tends to consistently produce considerably higher coliform values than MTF in water from Berg 2. While the tendency for considerably higher enumeration by Colilert in water from the other sites is evident in Table 4.2, the occurrence of such events is more sporadic and isolated. It is unclear why Colilert analyses resulted consistently in cases of severely higher coliform enumeration at Berg 2, but is probably attributable either to an unknown factor in the water or the nature of the bacteria in this area.

The coliform data set for the Plankenburg River is shown in Table 4.3. Mean, median and standard deviation values were calculated after exclusion of counts which fell outside the limits of the Colilert MPN table.

**Table 4.3** Coliform values (MPN.100 mL<sup>-1</sup>) for the Plankenburg River sites, enumerated by Colilert and MTF

Sample date	Plank 1		Plank 3	
	Colilert	MTF	Colilert	MTF
09/2009	129 015	49 000	182 215	49 000
10/2009	2 419 600	1 700 000	260 300	33 000
11/2009	81 640	79 000	*	*
12/2009	435 200	330 000	241 960	230 000
01/2010	1 046 200	230 000	410 600	220 000
02/2010	*	*	15 531	110 000
03/2010	150 000	130 000	161 600	33 000
04/2010	*	*	57 940	33 000
05/2010	228 200	79 000	1 986 300	49 000
<b>Mean</b>	641 408	371 000	414 556	94 625
<b>Median</b>	228 200	130 000	212 088	49 000

\* No data points were obtained for the specific site on this date

The results in Table 4.3 show that the mean value for Colilert was nearly double the mean value for MTF in water from Plank 1. The mean values for Plank 3 showed that the Colilert mean value was more than four times higher than the mean value for MTF. Five years of data on the Plankenburg River showed that the river at Plank 1 carries substantially more faecal pollution than

the river water at Plank 3 (Barnes, 2003). The differences between mean values for the two sites followed the same trend with respect to pollution levels which was observed for river water from the upper Berg River. This trend shows a closer agreement between Colilert and MTF coliform enumerations in river water which is highly faecally polluted, when compared to river water with a low level of faecal pollution.

The differences in median values depicted in Table 4.3 corresponded closely with the differences observed for mean values. The Colilert median value for Plank 1 was nearly double the MTF median value, while the Colilert median value for Plank 3 was around four times higher than the MTF median value. The results in Table 4.3 also show that considerably higher Colilert enumerations were isolated incidents for Plank 1 (September 2009; January and May 2010) as well as Plank 3 (September and October 2009; March and May 2010) during the sampling period.

The coliform data set for the Eerste and Lourens rivers is shown in Table 4.4. Mean, median and standard deviation values were calculated after exclusion of counts outside the limits of the Colilert MPN table.

**Table 4.4** Coliform values (MPN.100 mL<sup>-1</sup>) for sites in the Eerste and Lourens Rivers, enumerated by Colilert and MTF

Sample date	Eerste		Lourens	
	Colilert	MTF	Colilert	MTF
07/2009	*	*	129 015	49 000
08/2009	77 010	13 000	**	**
09/2009	4 271	490	7 734	2 700
10/2009	178 500	79 000	**	**
11/2009	7 701	330	**	**
12/2009	29 090	4 900	**	**
01/2010	9 330	6 400	**	**
02/2010	1 112	2 200	**	**
03/2010	31 300	3 300	**	**
04/2010	9 580	3 100	**	**
05/2010	27 900	4 900	**	**
<b>Mean</b>	37 579	11 762	68 375	25 850
<b>Median</b>	18 740	4 100	68 375	25 850

\* No data points were obtained for the specific site on this date

\*\* Not sampled

The mean Colilert value for the Eerste River site was more than three times higher than the mean value for MTF. Since only one site was sampled in this river there are no results, from water with a higher or lower level of faecal pollution, which can be used to compare the hypothesised trend for closer agreement between the two methods in more polluted river water. It did appear

from the results that higher Colilert enumerations were fairly consistent in their occurrence for this site and that the difference between Colilert and MTF mean values was not a function of occasional high value spikes in Colilert values.

The conclusions from the results on the Lourens River should be drawn with caution due to the extremely small data set. On the first sampling date, Colilert produced a considerably higher coliform enumeration when compared with MTF. However, the coliform enumeration done by Colilert on water obtained on the second sampling date was within the same order of magnitude as the enumeration done by MTF. The only conclusion which can be drawn from this is that Colilert also tended for the two data points towards higher enumeration of coliforms than MTF, as was observed for all sites in both the upper Berg and the Plankenburg Rivers.

The results in this section show that Colilert consistently gave higher counts than MTF in the enumeration of coliforms for all rivers. This is in agreement with work reported in literature. In 1998, Eckner had already shown that Colilert showed a higher recovery of coliforms in 20.6% of 247 water samples tested, while MTF showed higher recovery in only 3.6% of samples. Research reported by Noble *et al.* (2004) showed that total coliform results with Colilert and Colilert-18 were nearly twice that of MTF in the analysis of coastal water, while Kämpfer *et al.* (2008) reported that Colilert recovered 80.0% more coliform isolates. Al-Turki and El-Ziney (2009) also found higher recovery rates of coliforms by Colilert (38.7%), when compared to MTF (20.0%), in drinking water from Saudi Arabia. Although not a MTF comparative study, the work done by Olstadt *et al.* (2007) reported that Colilert showed poor recovery rates for *Klebsiella*, but high recovery rates for *E. coli*, *Enterobacter*, *Citrobacter* and *Serratia* from spiked groundwater sources. These results indicate that Colilert outperforms MTF in the recovery of coliforms from water, and suggests that perspective of the disagreement should be revised to include the possibility that MTF tends towards lower enumeration of coliforms when compared to Colilert.

#### Determination of Colilert and MTF category agreement for coliforms

In order to carry out a preliminary comparison of the coliform counts obtained by the two methods, frequency tables were constructed to determine the extent of agreement of Colilert with MTF. The grey diagonal represents the value categories where Colilert and MTF agree. The diagonal tiers above and below the grey diagonal represent values where Colilert produced higher and lower values than MTF, respectively. The tiers flanking the grey diagonal represent values where Colilert values differed slightly from MTF values. As the tiers progress away from the grey diagonal, the difference between Colilert values and MTF values increase. It is also important to keep in mind that the agreement diagonal is described by *categories*, and that this is not explicit agreement of counts. The frequencies of coliform values, from the three sites in the upper Berg River (classified as “less polluted”), are shown in Table 4.5.

**Table 4.5** Frequency table for coliform values (MPN.100 mL<sup>-1</sup>) from the Berg River sites (n=29)

		Colilert			
		0-3 000	3 001-6 000	6 001-9 000	>9 000
MTF	0-3 000	2	3	4	6
	3 001-6 000	0	1	0	4
	6 001-9 000	0	0	1	1
	>9 000	0	0	0	7

The trend for higher coliform enumeration by Colilert, when compared to MTF, is observable in Table 4.5 where this method tends to have a high proportion of counts in higher categories, with 18 cases (62.1%) occurring above the grey diagonal. No production of lower values was observed for Colilert. It should also be noted that the agreement between the two methods is low, with only 11 cases (37.9%) of category agreement.

The frequencies of coliform counts, from the two sites in the Plankenburg River (classified as “more polluted”), are shown in Table 4.6.

**Table 4.6** Frequency table for coliform values (MPN.100 mL<sup>-1</sup>) from the Plankenburg River sites (n=15)

		Colilert			
		0-30 000	30 001-60 000	60 001-90 000	>90 000
MTF	0-30 000	0	0	0	0
	30 001-60 000	0	1	0	5
	60 001-90 000	0	0	1	1
	>90 000	1	0	0	6

The results in Table 4.6 indicate the same trend for Colilert to produce higher coliform counts than MTF was found for the Plankenburg River. However, values in higher categories with Colilert were found in six cases (40.0%), which is lower than the proportion found for the Berg River. In addition, one case (6.7%) saw Colilert producing a value in a lower tier category. The overall level of agreement between the two methods is higher than that of the Berg River total coliform data set, with eight cases (53.3%) of category agreement. This observation reflects the results shown in the previous section, which showed the possibility of better method agreement in river water with increased levels of faecal pollution.

The frequencies of coliform values from the one site in the Eerste River (categorised as “less polluted”) are shown in Table 4.7.

**Table 4.7** Frequency table for coliform values (MPN.100 mL<sup>-1</sup>) from the Eerste River site (n=10)

		Colilert			
		0-5 000	5 001-10 000	10 001-15 000	>15 000
<b>MTF</b>	0-5 000	2	2	0	3
	5 001-10 000	0	1	0	0
	10 001-15 000	0	0	0	1
	>15 000	0	0	0	1

The results in Table 4.7 show that Colilert also has a propensity towards producing higher values in water from the Eerste River. For this river the number of cases where values were found in higher categories with Colilert is six (60.0% of the data set). No tendency by Colilert to produce lower values was observed for this site. The level of agreement between the two methods was low, with only four cases (40.0%) of category agreement, similar to the agreement found for the Berg River. The low level of agreement is in keeping with the observation that the two methods showed better agreement in highly contaminated water, and explains the weak agreement found for this river which was categorised as “less polluted”. A frequency table was not drawn up for the Lourens River coliform counts, since there are only two data points.

To gain a better overview of the differences in enumeration of coliforms from different rivers with Colilert, Table 4.8 shows the percentage category agreement and percentages of cases occurring in higher and lower tiers for Colilert. It must be kept in mind that, for instance, the first higher tier indicates a slight difference in the enumeration of the two methods while the second and third tiers indicate larger and even larger differences, respectively.

**Table 4.8** Differences in category agreement for coliform values by Colilert vs. MTF<sup>†</sup>

	Lower tiers			Category agreement	Higher tiers		
	3 <sup>rd</sup> lower tier	2 <sup>nd</sup> lower tier	1 <sup>st</sup> lower tier		1 <sup>st</sup> higher tier	2 <sup>nd</sup> higher tier	3 <sup>rd</sup> higher tier
Berg	0.0	0.0	0.0	37.9	13.8	27.6	20.7
Plankenburg	6.7	0.0	0.0	53.3	6.7	33.3	0.0
Eerste	0.0	0.0	0.0	40.0	30.0	0.0	30.0

<sup>†</sup> Values in percentage of the total number of counts per river

The percentages in Table 4.8 indicate that Colilert tended, save for one instance, towards values in higher categories for all three rivers. There are, however, differences in the degree of discrepancy from MTF values. For the Berg River, nearly 50% of all values occurred in the second and third higher tier, with only 13.8% occurring in the first higher tier. Therefore, nearly half of the

values showed large or very large differences from those values enumerated with MTF and, in addition, the agreement level was poor.

For the Plankenburg, 6.7% of values occurred in the third lowest tier, which is the tier of greatest difference containing values for this river. Furthermore, while only 6.7% of values were in the first higher tier, nearly five times more values occurred in the second higher tier. Therefore, although it may be a small percentage, the values found in the third lowest tier are values which have the greatest difference from those enumerated by MTF. Although there is a lesser degree of difference, the largest percentage of “non-agreement” values occurs in the second highest tier, which also describes considerable difference from values generated by MTF. Still, the level of category agreement was fairly good for this river.

The percentages for the Eerste River show that an equal amount of values were found in the first and third higher tier. The data set (n=10) may have been too small to generate a demonstrable trend other than a tendency by Colilert to enumerate higher, however, it is interesting to note that nearly a third of all the counts occurred in the third highest tier, which indicates large differences with the values enumerated by MTF. Although it may also be attributable to the small data set, the level of agreement was poor for this river, although not as poor as that of the Berg River.

There are observable discrepancies in agreement level between the Plankenburg River, which was categorised as “more polluted”, and the upper Berg and Eerste Rivers, both categorised as “less polluted”. The results in Table 4.8 are confirmation of the earlier observation that improved agreement is found between the two methods when samples are more polluted with faecal matter, and that agreement decreases as pollution decreases. However, the table also illustrates that the direction of disagreement is usually towards higher values by Colilert, and that the severity of these disagreements are exacerbated by decreasing pollution levels. However, despite the occurrence of disagreement, the results are encouraging, because for a method used in the assessment of microbiological risk a tendency towards minor over-estimation is always preferable in comparison with a tendency towards slight under-estimation.

### *E. coli*

The *E. coli* data set for the Berg River is shown in Table 4.9. Mean, median and standard deviation values were calculated after exclusion of counts outside the limits of the Colilert MPN table. MTF-obtained values in bold are outside of the WHO guideline cut-off value of 1 000 faecal (thermotolerant) coliforms per 100 mL, set for water used in the irrigation of produce likely to be consumed raw (WHO, 1989).



**Table 4.9** *E. coli* values (MPN.100 mL<sup>-1</sup>) for the Berg River sites enumerated by Colilert and MTF

Sample date	Berg 1		Berg 2		Berg 3	
	Colilert	MTF	Colilert	MTF	Colilert	MTF
05/2009	*	*	73	34	801	110
07/2009	245	180	158	28	1 529	<b>1 100</b>
08/2009	932	220	254	140	5 172	<b>1 100</b>
09/2009	1 333	<b>1 100</b>	1 887	580	2 569	<b>3 100</b>
10/2009	1 482	280	474	230	7 350	<b>1 400</b>
11/2009	2 495	<b>1 100</b>	5 794	<b>1 100</b>	12 033	<b>23 000</b>
12/2009	910	280	144	130	448	790
01/2010	52	79	20	33	1 046	330
02/2010	*	*	63	430	331	310
03/2010	20	8	62	79	934	<b>2 300</b>
04/2010	31	22	63	130	2 603	<b>2 300</b>
<b>Mean</b>	833	363	817	265	3 165	3 258
<b>Median</b>	910	220	144	130	1 529	1 100

\* No data points were obtained for the specific site on this date

The mean values calculated for Colilert were more than two and nearly four times higher than MTF mean values for Berg 1 and Berg 2, respectively. Interestingly, the mean value for Colilert at Berg 3 was slightly lower than the mean value for MTF. These results are indicative of a trend, similar to that observed for coliforms, by Colilert to enumerate higher than MTF when *E. coli* is analysed. In addition, the two sites, Berg 1 and Berg 2, which are considered to be markedly less polluted than Berg 3 showed weaker enumeration agreement between Colilert and MTF than did Berg 3. This observation indicates the possibility that the improved agreement of Colilert with MTF in more polluted water may be a phenomenon found in the enumeration of both coliforms and *E. coli*.

The median values for the three sites indicated some difference for the two methods at Berg 1, while the median values for Berg 2 and Berg 3 were similar for the two methods. The primary cause of the difference between mean values for Berg 1 was presumably the isolated case of considerably higher enumeration by Colilert (October 2009). When examining the results for the other two sites, it is clear that very few cases of considerably higher counts with Colilert were found and that the differences in means were attributable to consistent but slightly higher enumerations by Colilert, in most cases. Interestingly, the occurrence of higher enumeration by MTF was also observed more frequently for these sites.

Aside from the methodological comparisons done here, the instances of exceeding of the WHO guideline for irrigation water used on crops likely to be eaten raw shown in Table 4.9 are

concerning. The upper Berg River is an important source of irrigation water for the production of crops (DWAF, 2004) destined for the local and export market, and the risk of irrigating MPFs with water of this microbiological quality could pose health risks to the consumer, as well as food handlers in the supply chain. The reason why *E. coli* results could be evaluated using a guideline based on thermotolerant coliforms is due to the fact that *E. coli* forms part of the thermotolerant coliform group, and that exceeding of the guideline by one member of the thermotolerant coliform group indicates potentially even greater transgression of the guideline by the entire group.

The *E. coli* data set for the Plankenburg River is shown in Table 4.10. Mean, median and standard deviation values were calculated after exclusion of values outside the limits of the Colilert MPN table. MTF-obtained values in bold exceeded the WHO guideline of less than 1 000 faecal (thermotolerant) coliforms per 100 mL, set for water used in the irrigation of produce likely to be consumed raw (WHO, 1989).

**Table 4.10** *E. coli* values (MPN.100 mL<sup>-1</sup>) for the Plankenburg River sites, enumerated by Colilert and MTF

Sample date	Plank 1		Plank3	
	Colilert	MTF	Colilert	MTF
08/2009	44 538	<b>330 000</b>	8 305	<b>21 000</b>
09/2009	5 754	<b>49 000</b>	8 051	<b>22 000</b>
10/2009	173 290	<b>1 300 000</b>	46 110	<b>33 000</b>
11/2009	12 997	<b>49 000</b>	23 330	<b>460 000</b>
12/2009	17 329	<b>79 000</b>	5 172	<b>130 000</b>
01/2010	38 730	<b>49 000</b>	4 100	<b>2 300</b>
02/2010	19 863	<b>170 000</b>	754	<b>2 100</b>
03/2010	21 300	<b>79 000</b>	2 000	<b>1 800</b>
04/2010	579 400	<b>280 000</b>	740	<b>1 100</b>
05/2010	34 500	640	10 900	<b>22 000</b>
<b>Mean</b>	94 770	238 564	10 946	69 530
<b>Median</b>	27 900	79 000	6 612	21 500

The mean values shown in Table 4.10 indicate, contrary to the previous results, that Colilert tended towards considerably lower *E. coli* enumeration when compared to MTF. The mean value for Colilert at Plank 1 was less than half of the mean value for MTF, while the mean value for Colilert at Plank 3 was nearly seven times lower than the mean value for MTF. This is the first occurrence of such a trend in this comparative study. The reasons for this surprising reaction are unclear. However, this trend is possibly linked to the composition of the water, since this river flows past the Plankenburg industrial area. The nature of the effluent which reaches the river is complex and unknown, but it is possible that some chemical agent in the water caused interference with the detection of *E. coli* by Colilert.

The difference in median values for Plank 1 were even greater than the difference in mean values for this site, with the Colilert median value nearly three times lower than the MTF median value. In contrast, the median value for Colilert at Plank 3 was only three times lower than the median value for MTF. The difference in mean values was probably attributable to considerably higher MTF values for Plank 1 (August, September and October 2009; February 2010) and Plank 3 (August, September, November and December 2009; February and April 2010).

Despite the fact that the two Plankenburg sites are not sites for the irrigation of produce, the water from the Plankenburg River represents a tributary of the Eerste River catchment, which provides irrigation water for agricultural activities downstream from its confluence with the Plankenburg. Therefore, the exceeding of the WHO guideline by as many as 1 300 times (Plank 1, October 2009) should remain a point of concern for the irrigation of produce likely to be eaten raw. In addition, the results in Table 4.10 indicate that the water from the two sites only conformed to this guideline in one instance. The presence of such heavy faecal contamination represents an alarming health risk to consumers of produce irrigated with this water further downstream. In addition, these levels of faecal pollution could also endanger the health of riverbank dwellers that frequently use this water for domestic purposes, as well as individuals coming into contact with the river through recreational use (WHO, 2003).

The *E. coli* data set for the Eerste and Lourens Rivers is shown in Table 4.11. Mean, median and standard deviation values were calculated after exclusion of values outside the limits of the Colilert MPN table. MTF-obtained values in bold exceed the WHO guideline cut-off value of 1 000 faecal (thermotolerant) coliforms per 100 mL, set for water used in the irrigation of produce likely to be consumed raw (WHO, 1989).

It can be seen in Table 4.11 that the mean values for the Eerste River, a “less polluted” river, showed the same trend as that found for the Plankenburg River; with the MTF mean nearly 1.5 times higher than the mean value for Colilert. This difference was, however, primarily attributable to consistent and slightly higher enumeration by MTF and not due to one isolated instance of a considerably higher value. These results are surprising, however, since the Eerste River is considered to be a “less polluted” river. As with the results for coliforms, the conclusions for the two *E. coli* enumeration results of the Lourens River should be drawn carefully. On both sampling occasions, MTF produced considerably higher *E. coli* values (49 000 vs 5754 and 1 300 vs 601 *E. coli* MPN.100 mL<sup>-1</sup>) when compared to Colilert. This is in agreement with the results reported for the Plankenburg River, where MTF also tended to enumerate higher than Colilert. It is possible that this phenomenon may be linked to the pollution level, since both these rivers are classified as “more polluted”.

**Table 4.11** *E. coli* values (MPN.100 mL<sup>-1</sup>) for sites in the Eerste and Lourens Rivers, enumerated by Colilert and MTF

Sample date	Eerste		Lourens	
	Colilert	MTF	Colilert	MTF
07/2009	*	*	5 754	<b>49 000</b>
08/2009	329	350	**	**
09/2009	209	490	601	<b>1 300</b>
10/2009	54 750	<b>79 000</b>	**	**
11/2009	350	330	**	**
12/2009	313	<b>1 700</b>	**	**
01/2010	100	170	**	**
02/2010	41	790	**	**
03/2010	1 000	330	**	**
04/2010	100	230	**	**
05/2010	**	**	**	**
<b>Mean</b>	6 355	9 266	3 178	25 150
<b>Median</b>	313	350	3 178	25 150

\* No data points were obtained for the specific site on this date

\*\* Not sampled

The *E. coli* counts for the Eerste River site showed only two instances of exceeding the WHO guideline. The *E. coli* numbers for October 2009 are, however, extremely high and represent a considerable risk both for humans coming into contact with the water through domestic or recreational contact as well as consumers of produce irrigated with this water. The transgression of WHO guidelines by the Lourens River, on the two sampling dates, indicates that sewage intrusion is taking place along the course of the river, and is also an indication of the pollution loads which are deposited into the ocean at False Bay. If this intrusion is taking place upstream from agricultural activities which use the Lourens River as irrigation source, this river could also pose a threat to the health of the consumers of minimally processed foods.

The *E. coli* results in this section show that while Colilert tended towards higher enumeration for the upper Berg River, this method was consistently lower when compared to MTF for the Plankenburg, Eerste and Lourens Rivers. The latter results are in agreement with the work done by Noble *et al.* (2004), which reported lower comparative Colilert values when enumerating *E. coli* from coastal waters. These workers, however, compared faecal (thermotolerant) coliforms MTF results with *E. coli* Colilert results, and concluded that the lower Colilert results were attributable to the enumeration of the entire thermotolerant coliform group by MTF. This was not the case for the present study, and cannot be used to explain the lower enumeration by Colilert. However, a comparative study done in Buraidah, Saudi Arabia, found that Colilert had a

significantly higher ( $p>0.05$ ) recovery rate (13.7%) when compared to MTF (7.5%) in the enumeration of *E. coli* in drinking water (Al-Turki & El-Ziney, 2009). This finding may be able to clarify the different results found for the upper Berg River, since drinking water should carry very low faecal bacteria loads. It has also been demonstrated in this study that the faecal bacteria counts in the Berg River is lower than that found in the other rivers. It is possible, therefore, that a high level of pollution interferes with the recovery rate of *E. coli* with Colilert. This was also observed in the work by Olstadt *et al.* (2007), who found that the recovery rate of *E. coli* by Colilert-18 decreased when inoculum concentrations of five species of total coliforms used in spiked groundwater were increased from 1-10 to 50-100 bacteria.

#### Determination of Colilert and MTF category agreement for *E. coli*

In order to carry out a preliminary comparison of the coliform counts obtained by the two methods, frequency tables were constructed to determine the extent of agreement of Colilert with MTF. The grey diagonal and various diagonal tiers represent the same agreement or differences in values, as explained for the coliform frequency tables. The frequencies of *E. coli* values, from the three sites in the Berg River (classified as “less polluted”), are shown in Table 4.12.

**Table 4.12** Frequency table for *E. coli* values (MPN.100 mL<sup>-1</sup>) from the Berg River sites (n=31)

		Colilert			
		0-400	401-800	801-1 200	>1 200
MTF	0-400	12	1	4	1
	401-800	1	1	0	1
	801-1 200	0	0	0	5
	>1 200	0	0	1	4

As was observed in the previous section, it can be seen in Table 4.12 that Colilert tended to produce higher values and had 12 cases (38.7%) of values in higher categories for the Berg River. In two cases (6.5%), Colilert had values in lower categories. The level of agreement between the two methods is fair, but considerably lower than that of the entire data set, with 17 cases (54.8%) of category agreement.

The frequencies of *E. coli* values, from the two sites in the Plankenburg River (classified as “more polluted”), are shown in Table 4.13.

**Table 4.13** Frequency table for *E. coli* values (MPN.100 mL<sup>-1</sup>) from the Plankenburg River sites (n=20)

		Colilert			
		0-20 000	20 001- 40 000	40 001- 60 000	>60 000
MTF	0-20 000	4	1	0	0
	20 001- 40 000	3	0	1	0
	40 001- 60 000	2	1	0	0
	>60 000	3	2	1	2

The results for the Plankenburg River show, in contrast to the *E. coli* data set for the upper Berg River, that Colilert tended towards lower values than MTF with 12 cases (60.0%) of values in lower categories. Only two cases (10.0%) of values in higher categories occurred for this river. The level of agreement between the two methods was much lower than the agreement found for the upper Berg River, with only six cases (30.0%) of category agreement.

The frequencies of *E. coli* values, from the one site in the Eerste River (classified as “less polluted”), are shown in Table 4.14.

**Table 4.14** Frequency table for *E. coli* values (MPN.100 mL<sup>-1</sup>) from the Eerste River site (n=9)

		Colilert			
		0-200	201-400	401-600	>600
MTF	0-200	1	0	0	0
	201-400	1	2	0	1
	401-600	0	1	0	0
	>600	1	1	0	1

The same trend observed in Table 4.13 can be seen, to a lesser extent, in Table 4.14, where Colilert once again tended to produce lower values. Four cases (44.4%) of values in lower categories can be seen, in comparison to only one case (11.1%) of a value in a higher category. The level of agreement between the two methods is poor, but better than the 44.4% category agreement for the Plankenburg River.

A frequency table was not drawn up for the Lourens River *E. coli* values, since there are only two data points. However, Colilert values were lower than MTF counts for both July and September 2009. Although these results cannot be effectively used in the evaluation of Colilert against MTF or in the investigation of the performance of Colilert with water samples of varying degrees of pollution, they do indicate that the trend observed for the Plankenburg and Eerste Rivers was reflected in water from this site. The reason for the lower enumeration of *E. coli* by

Colilert remains unclear. Since this trend was identified in three separate river systems, the likelihood that the origin of the discrepancy lies with these rivers is small. It is more likely that the observed differences are due to physiological or concentration differences in bacteria of the upper Berg River. No such differences were investigated during this study, but it is possible that a high proportion of anaerogenic strains (strains unable to produce gas from lactose) of *E. coli* were present and remained undetected by MTF. Alternatively, Colilert may have yielded higher *E. coli* numbers due to low concentrations of competing coliforms.

To gain a better overview of the differences in enumeration of *E. coli* from different rivers with Colilert, Table 4.15 summarises the percentage agreement and percentages of cases occurring in higher and lower tiers for Colilert.

**Table 4.15** Differences in category agreement for *E. coli* values by Colilert vs. MTF<sup>†</sup>

	Lower tiers			Category agreement	Higher tiers		
	3 <sup>rd</sup> lower tier	2 <sup>nd</sup> lower tier	1 <sup>st</sup> lower tier		1 <sup>st</sup> higher tier	2 <sup>nd</sup> higher tier	3 <sup>rd</sup> higher tier
Berg	0.0	0.0	6.5	54.8	19.4	16.1	3.2
Plankenburg	15.0	20.0	25.0	30.0	10.0	0.0	0.0
Eerste	11.1	11.1	22.2	44.4	0.0	11.1	0.0

<sup>†</sup> Values in percentage of the total number of counts per river

As can be seen in Table 4.17, Colilert was not as prone to consistently producing values in higher categories as was the case with coliforms. For the Berg River, however, the trend seemed to remain, with nearly 40% of values occurring in the higher tiers. Only 3.2% of values occurred in the third higher tier, nearly 20% occurred in the first higher tier and 6.5% of values are found in the first lower tier. Therefore, the percentage of values occurring in tier with a large or very large difference from the corresponding MTF count amounted to less than 20.0%. Additionally, the level of agreement was fairly good for this river.

The Colilert values from the Plankenburg River showed a definite tendency to be lower than those enumerated by MTF. These values found in the lower tiers amounted to 60%, with 35% occurring in the tiers describing large and very large differences with MTF. Only 10% occurred in the first higher tier. Therefore, most values in this data set were lower than those enumerated by MTF and, furthermore, a considerable amount of these differed greatly from those generated by MTF. The level of agreement for this river was also extremely poor.

The Eerste River showed a similar pattern to that found for the Plankenburg River, albeit not as severe. Values occurring in lower tiers amounted to 44.4%, but only half of those occurred in the tiers describing large and very large differences when compared to MTF values. The values found in higher tiers, however, were all located in the second higher tier which indicates large difference with the corresponding MTF values. The level of agreement was also poor, but remains



somewhat higher than that found for the Plankenburg River. Once again, it should be noted that the data set was extremely limited ( $n=9$ ) and conclusions should be made with the appropriate caution.

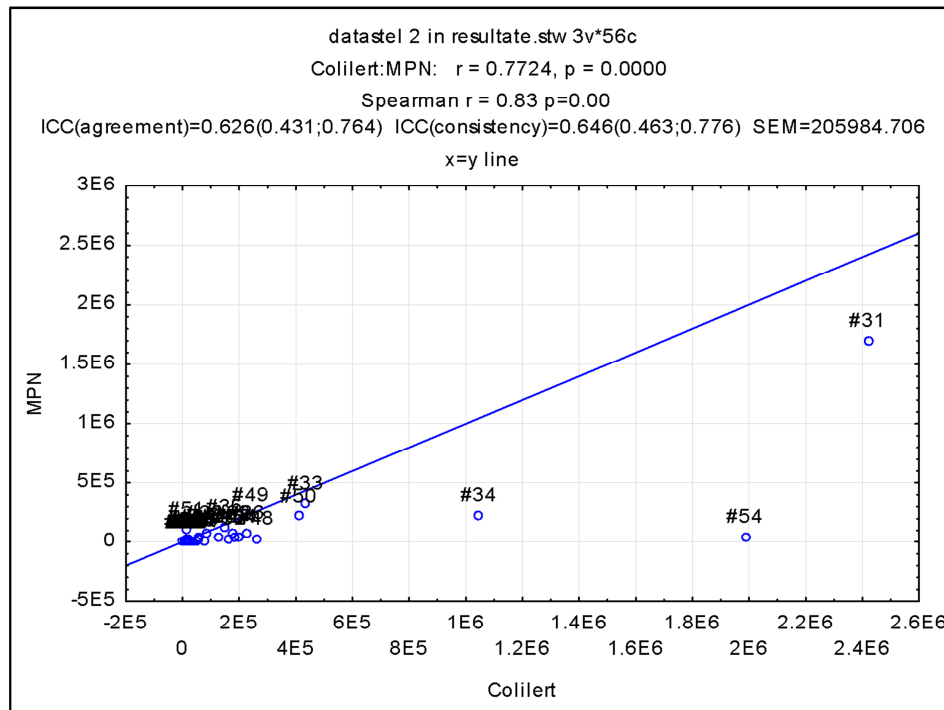
Unlike the results for the enumeration of coliforms, no definite trends could be identified from the results for the enumeration of *E. coli*. It appears, however, that river water from the upper Berg River resulted in the highest level of agreement for the two methods. However, when disagreement occurred, the direction was towards higher enumeration by Colilert. The agreement levels for the Plankenburg and Eerste Rivers with this data set were poor, and disagreement tended to favour lower enumeration by Colilert. The direction of disagreement for the Plankenburg and Eerste Rivers are somewhat concerning, because the lower enumeration by Colilert alludes to a propensity for under-estimation of *E. coli*. This is not ideal, since risk assessment procedures benefit more from over-estimation and the associated increase in preventative measures, rather than under-estimation and the inadvertent reckless decision making which can result.

#### 4.4.2. Method comparison

From the preliminary frequency table results, and especially the discrepancies between Colilert and MTF, it is clear that formal statistics should be employed to make accurate conclusions regarding the efficacy of Colilert as a substitute for MTF. This is necessary for both coliforms and *E. coli* values.

##### Coliforms

The linear correlation between MPN and Colilert values for the analysis of coliforms for the four-river data set is illustrated in Fig. 4.4 on the next page. It can be seen in the figure that there is a fairly good correlation between the values obtained by Colilert and those generated by MTF. Even with some data outliers, the Spearman rank correlation coefficient is 0.83. The Spearman  $r^2$  is 0.69, which indicates that the regression line will approximate real data points correctly in 69.0% of cases. These findings are similar to those reported by Noble *et al.* (2004), who obtained a correlation coefficient of 0.91 between Colilert-18 and MTF for the enumeration of coliforms. However, this work was done on coastal waters which would have differed from river water in its composition and microbiological population. In addition, Pearson correlation coefficients were calculated, which influenced to a larger extent by data outliers than Spearman rank correlation coefficients.

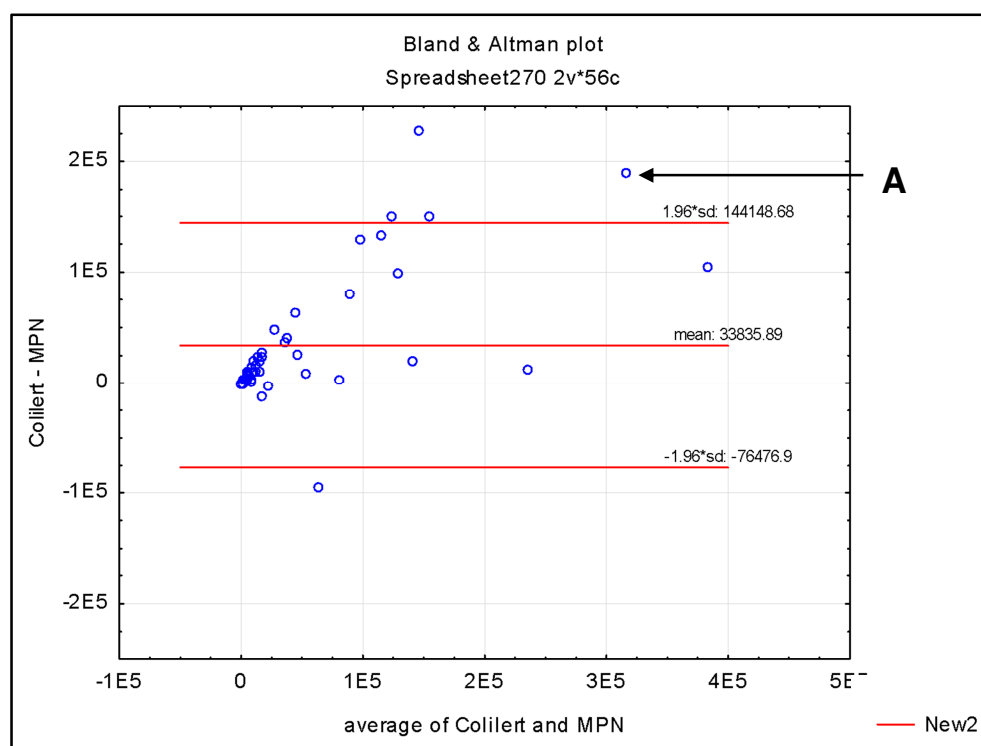


**Figure 4.4.** Scatterplot and regression line for Colilert vs. MTF (referred to as MPN) for coliform enumeration

The Spearman correlation found here does not agree well with the results of Al-Turki and El-Ziney (2009), who obtained a Spearman rank correlation coefficient of 0.59 when comparing Colilert-18 with MTF in the enumeration of coliforms from Saudi Arabian drinking water. The differences between the two water sources could explain these discrepancies. Drinking water is typically of a good microbiological quality, while the pooled results of four rivers used in this statistical comparison included three rivers which were shown to carry heavy faecal pollution loads.

The ICC for agreement is 0.626, ICC for consistency is 0.646 and the Standard Error of Measurement (SEM) is 205984.7. The ICC values show that no mathematical correction factor is necessary for Colilert to substitute MTF for the analysis of coliforms. These results indicate that if Colilert is to be used instead of MTF in the analysis of water from the upper Berg, Plankenburg, Eerste or Lourens Rivers, the coliform results obtained by this method can be used as surrogate values for MTF coliform results without any mathematical adjustment. In addition, these coliform results would have a likelihood of 69.0% of estimating the MTF value correctly.

The Bland and Altman scatterplot comparing Colilert and MPN values for coliforms at varying pollution levels is given as Fig. 4.5. The “true” pollution levels were taken as the average of Colilert and MTF enumerations, and are shown on the x-axis.

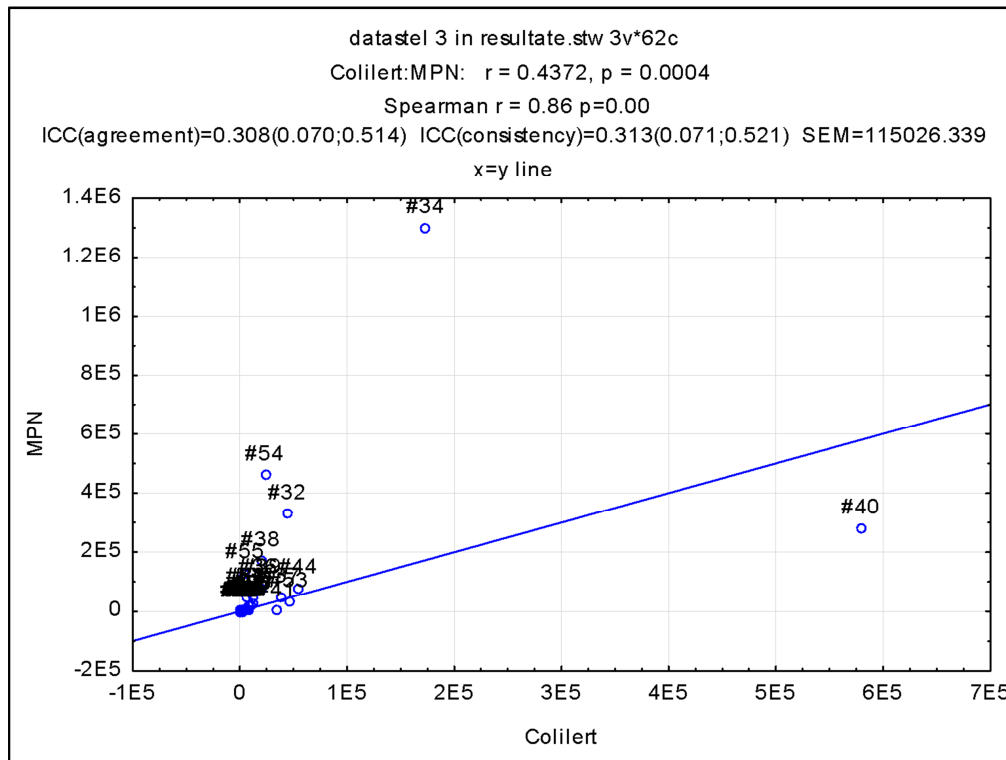


**Figure 4.5.** Bland and Altman scatterplot for comparison of Colilert and MTF (referred to as MPN) for the enumeration of coliforms at varying pollution levels

Contrary to the observational conclusions drawn from the coliform enumeration results, the Bland and Altman scatterplot shown in Fig. 4.5 indicate that Colilert enumerations agreed well with MTF enumerations when pollution levels were low (0 to ca. 100 000 coliforms MPN.mL<sup>-1</sup>). Furthermore, the scatterplot shows that an increase in the “true” pollution level (x-axis) will result in Colilert enumerations which are increasingly higher than MTF enumerations. For example, a coliform concentration of around 300 000 MPN.100 mL<sup>-1</sup> will lead to a Colilert enumeration that is approximately 200 000 MPN.100 mL<sup>-1</sup> higher than the MTF enumeration (corresponding to point A indicated on the plot). Consequently, as the faecal pollution level of a river increases, so the inaccuracy of the Colilert enumeration of coliforms will increase in the direction of over-estimation.

### *E. coli*

The linear correlation between MPN and Colilert values for the analysis of *E. coli* for the four-river data set is illustrated in Fig. 4.6.

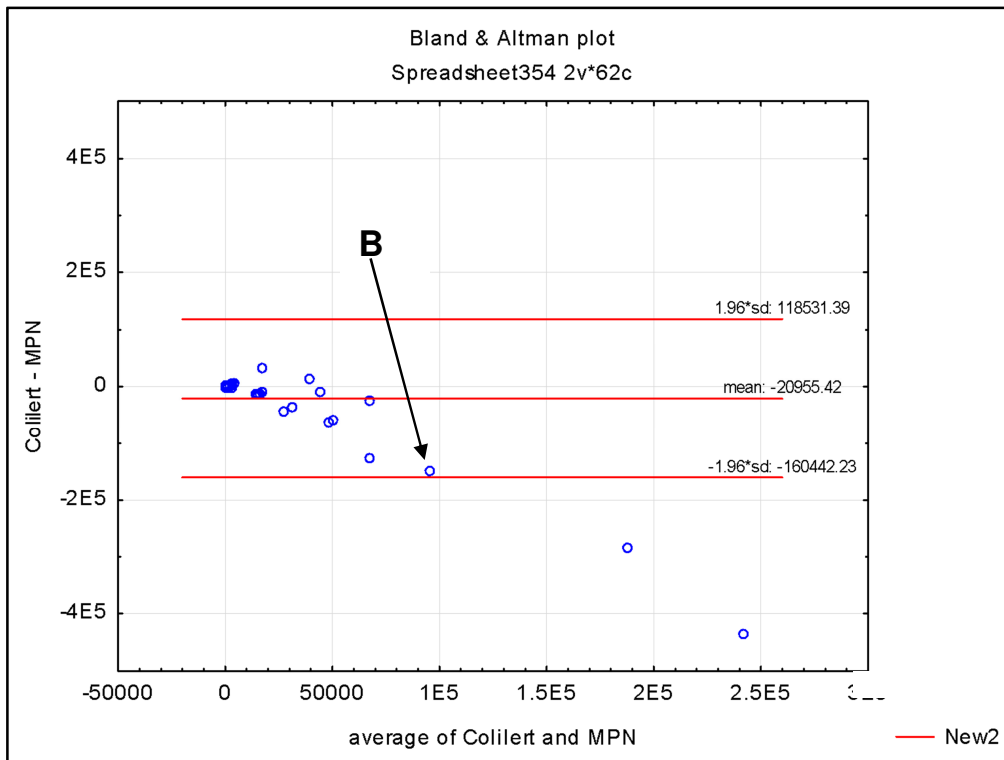


**Figure 4.6.** Scatterplot and regression line for Colilert vs. MTF (referred to as MPN) for *E. coli* enumeration

It can be seen in the figure, there is a good correlation between the values generated by Colilert and those generated by MTF. Even with some data outliers, the Spearman rank correlation coefficient is 0.86. The Spearman  $r^2$  is 0.74, which indicates that the regression line will approximate real data points correctly in 74% of cases. The correlation coefficient obtained here is similar to that of Noble *et al.* (2004) and Al-Turki and El-Ziney (2009), who found correlation coefficients of 0.79 and 0.80, respectively, when comparing the enumeration of *E. coli* with Colilert and MTF, despite the differences in water samples and the use of faecal (thermotolerant) coliform MTF results in the study done by Noble *et al.* (2004). The similarity of these results suggest that Colilert compares favourably with MTF in the enumeration of *E. coli* regardless of the type of water sample used, and could be used as an alternative method for this purpose.

The ICC for agreement is 0.308, ICC for consistency is 0.313 and the Standard SEM is 115026.3. No correction factor is therefore necessary for Colilert to substitute MTF for the analysis of *E. coli*. These results indicate that if Colilert is to be used instead of MTF in the analysis of water from the upper Berg, Plankenburg, Eerste or Lourens Rivers, the *E. coli* results obtained by this method can be used as surrogate values for MTF *E. coli* results without any mathematical adjustment. In addition, these *E. coli* results would have a likelihood of 74.0% of estimating the MTF value correctly.

The Bland and Altman scatterplot comparing Colilert and MPN values for *E. coli* at varying pollution levels is given as Fig. 4.7. The “true” pollution levels were taken as the average of Colilert and MTF enumerations, and are shown on the x-axis.



**Figure 4.7.** Bland and Altman scatterplot for comparison of Colilert and MTF for *E. coli* at varying pollution levels

The Bland and Altman scatterplot shown in Fig. 4.7 indicate that Colilert enumerations agreed well with MTF enumerations when pollution levels were low (0 to ca. 50 000 *E. coli* MPN.100 mL<sup>-1</sup>). In contrast to Fig. 4.5, however, the scatterplot shows that an increase in the “true” pollution level (x-axis) will result in Colilert enumerations which are increasingly lower than MTF enumerations. For example, an *E. coli* concentration of around 100 000 MPN.100 mL<sup>-1</sup> will lead to a Colilert enumeration that is nearly 200 000 MPN.100 mL<sup>-1</sup> lower than the MTF enumeration (corresponding to point B indicated on the plot). Consequently, as the faecal pollution level of a river increases, so the inaccuracy of the Colilert enumeration of *E. coli* will increase in the direction of under-estimation.

## 4.5. CONCLUSIONS

### 4.5.1. Colilert as rapid alternative for MTF in the enumeration of coliforms

The preliminary results of the evaluation of Colilert as an alternative method for MTF indicated that Colilert mostly showed low (upper Berg River) to intermediate (Plankenburg River) levels of agreement with MTF enumerations and that the disagreement between the two methods were primarily due to a tendency by Colilert to enumerate higher coliform values than MTF, or a tendency by MTF to enumerate lower coliform values than Colilert. The latter perspective was enforced by literature which showed high recovery rates of coliforms by Colilert (Olstadt *et al.*, 2007), but did not compare them to MTF recovery of coliforms. These findings necessitate further investigation into the accuracy of enumeration by MTF, to determine whether the disagreement between the two methods is caused by poor recovery of coliforms by MTF.

When statistics were employed it was shown by the Bland and Altman scatterplot that Colilert agreed well with MTF at lower levels of pollution with coliforms (0 to 100 000 coliforms MPN.100 mL<sup>-1</sup>) and became increasingly prone to error towards higher enumeration as pollution levels increased. The differences in the two conclusions relating to coliform pollution levels are probably attributable to the definition of “pollution level” against which Colilert was compared. In the discussion of results, the MTF enumeration value was seen as the “true” pollution level, while formal statistics used the average value obtained by the two methods as the “true” value. The latter assumption is likely to be the more correct of the two, and therefore it is accepted that Colilert agrees well with MTF in water with coliform levels between 0 and 100 000 MPN.100 mL<sup>-1</sup> and becomes increasingly erroneous as levels increase beyond 100 000 MPN.100 mL<sup>-1</sup>.

The application of statistics to the coliform data set also showed a fair correlation between the enumeration values of Colilert and MTF, presumably because most of these values occurred between 0 and 100 000 coliforms MPN.100 mL<sup>-1</sup>. The  $r^2$  value obtained by Spearman rank correlation coefficient was acceptable enough ( $r^2=0.69$ ) to recommend that Colilert can be used for the rapid detection of coliforms in water from rivers in the Western Cape. The 31.0% of variation in coliform enumeration by MTF which cannot be explained by Colilert enumeration of this group represents a risk which is low enough when compared to the benefits of obtaining rapid results (data 18 hours later) which can result in early preventative action to stop contact or irrigation with river water downstream.

### 4.5.2. Colilert as rapid alternative for MTF in the enumeration of *E. coli*

The preliminary results of the evaluation of Colilert as an alternative method for MTF enumeration of *E. coli* showed that low (Plankenburg River) to intermediate (upper Berg River) levels of agreement between the two methods were obtained. When disagreement occurred between the methods for samples from the upper Berg River, Colilert tended towards higher enumeration than

MTF. In contrast, disagreement between the methods for water from the Plankenburg, Eerste and Lourens Rivers resulted in Colilert tending towards lower enumeration when compared to MTF.

It is recommended that a more in-depth study, focusing on the influence of the pH of the river water, is done to determine whether pH could affect the lower *E. coli* enumerations obtained by Colilert. Methylumbelliferone (MUF), the fluorogenic product of MUG hydrolysis, has been reported to be highly fluorogenic at high pH values (ca. 9.5-10.5) (Fang *et al.*, 1995), and Manafi (2000) stated that an alkali can be added to samples with a low pH to assist in the observation of fluorescence. Therefore, it is possible that the low pH of the river water may have contributed towards the trend by Colilert to enumerate lower than MTF.

The application of statistics to the *E. coli* data set reveal, through the use of a Bland and Altman scatterplot, that Colilert values agreed well with MTF values in the range between 0 and 50 000 *E. coli* MPN.100 mL<sup>-1</sup>. Interestingly and in contrast with the results for coliforms, Colilert tended strongly towards lower enumeration than MTF when the *E. coli* numbers exceeded 50 000 MPN.100 mL<sup>-1</sup>. These results are a reflection of the trend observed in water samples from the Plankenburg, Eerste and Lourens Rivers and are further evidence to support the theory that the upper Berg River differed due to its lower faecal pollution levels. Despite these discrepancies, the results of the formal statistics clearly indicate that Colilert can be used confidently, instead of MTF, in water samples with *E. coli* levels ranging from 0 to 50 000 MPN.100 mL<sup>-1</sup>.

The Spearman rank correlation coefficient resulted in an  $r^2$  value of 0.74, which is considered fair in the microbiological context (Ferrati *et al.*, 2005; Tavoraro *et al.*, 2005). This fair correlation is probably attributable to the fact that most of the samples had levels of *E. coli* within the range of 0-50 000 MPN.100 mL<sup>-1</sup>. In addition, the  $r^2$  value is high enough to recommend the use of Colilert as a rapid alternative for MTF in water analyses where immediate action based on the results is imperative. However, the 26.0% risk of inaccurately representing the MTF enumeration will more than likely manifest in an under-estimation by Colilert, a point which needs to be kept in mind when the threat posed for the irrigation of fresh produce is assessed.

#### 4.5.3. Performance of Colilert at varying levels of river pollution

The Bland and Altman scatterplots showed that Colilert enumeration compared well with MTF enumeration when coliform levels were between 0 and 100 000 MPN.100 mL<sup>-1</sup>, or when *E. coli* levels were between 0 and 50 000 MPN.100 mL<sup>-1</sup>. When coliform levels increased beyond the aforementioned range, Colilert exhibited an increasing tendency to enumerate coliforms higher than MTF. Conversely, an increase of *E. coli* numbers beyond 50 000 MPN.100 mL<sup>-1</sup> would result in a tendency by Colilert to produce lower *E. coli* enumerations than MTF.

These results show that river water samples with low to intermediate levels of faecal pollution can be confidently analysed, for coliforms and *E. coli*, with Colilert instead of MTF. However, in the analysis of highly faecally polluted water (> 100 000 coliforms MPN.100 mL<sup>-1</sup> or > 50 000 *E. coli* MPN.100 mL<sup>-1</sup>) it should be kept in mind that the results for coliforms are most likely



over-estimated, while the results for *E. coli* are probably under-estimated. In addition, the observation that Colilert tends towards increasingly inaccurate enumerations above certain levels of both coliforms and *E. coli* indicates that this method may have upper operational limits above which enumerations are no longer reliable.

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## CHAPTER 5

### PHENOTYPIC CHARACTERISATION AND IDENTIFICATION OF ATYPICAL AND TYPICAL ORGANISMS ISOLATED FROM THE MULTIPLE TUBE FERMENTATION METHOD

#### 5.1. ABSTRACT

Organisms which were isolated from positive and negative reactions in steps of the multiple tube fermentation (MTF) method were characterised using various routine biochemical tests as well as *E. coli*/coliform chromogenic agar. These isolates were subsequently identified using both the routine Analytical Profile Index (API) 20E system as well as a novel technology based on matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). The identification results and the MTF reactions were used to determine whether the isolates contributed to under- or over-estimation of coliform and *Escherichia coli* numbers by this method. The results of this study indicated that the greatest problem associated with the MTF enumeration of coliforms was under-estimation of this group due to a large proportion of coliforms (85.7% and 54.5% of isolates from the lauryl sulphate tryptose (LST) and brilliant green lactose bile (BGLB) steps, respectively) which cannot produce gas from lactose. The genera implicated here were *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Morganella*, *Providencia*, *Raoultella* and *Serratia*. Problematic isolates were less prevalent in media enumerating *E. coli*, but isolates causing over-estimation (17.1% and 7.7% of isolates from *E. coli* (EC) broth with 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG) and Levine-eosin methylene blue (L-EMB) steps, respectively) were identified. The genera implicated were *Cronobacter*, *Enterobacter*, *Klebsiella*, *Proteus* and *Serratia*. *E. coli* isolates causing under-estimation (9.8% and 38.5% of isolates from the EC with MUG and L-EMB steps, respectively) were also identified; and the associated negative reactions were either a failure to produce gas from lactose or fluorescence with MUG, or both, as well as the production of not metallic green (NMG) colonies. The results generated by the API 20E and MALDI-TOF MS systems showed that while API 20E exhibited limitations in its ability to identify environmental isolates (18.9% of isolates were reassigned to different genera by MALDI-TOF MS), the results from the API 20E method were acceptably reliable when considering the higher feasibility of this method for routine analysis. The lack of discriminatory ability of *E. coli*/coliform chromogenic agar based on the detection of  $\beta$ -D-galactosidase and  $\beta$ -D-glucuronidase was also demonstrated here, with 23.7% of *E. coli* isolates not distinguished from coliforms and 32.4% of coliform isolates not distinguished from non-coliforms.

## 5.2. INTRODUCTION

In spite of the emergence of improved rapid methods for the detection and enumeration of faecal indicator bacteria, the MTF method remains one of the important traditional methods for the detection and enumeration of coliforms (Rompré *et al.*, 2002) and *E. coli* (Prats *et al.*, 2008) from both food and water sources. The South African Water Quality guidelines for irrigation water still prescribe the use of either membrane filtration or the MTF method for the analysis of water sources (DWAF, 1996). The strength of this method lies in its high accuracy attributable to various hurdles in the discrete steps of the protocol and ability to statistically semi-quantify the number of faecal indicator organisms present in 100 mL of sample through the utilisation of most probable number (MPN) tables (Edberg & Edberg, 1988; Christensen *et al.*, 2002). It is for these reasons that the MTF method is still preferred over more novel methods, despite being expensive, labour intensive and only producing final results after four days (Maheux *et al.*, 2008).

Problems with the MTF method have, however, been reported. One of the most troublesome observations has been the presence of anaerogenic strains of coliforms and especially *E. coli* (Meadows *et al.*, 1980; Evans *et al.*, 1981b; Leclerc *et al.*, 2001) which fail to show gas production in the media (Edberg *et al.*, 1988). LeChevallier *et al.* (1983) showed that 91.5% of the strains they found to be anaerogenic in lauryl tryptose broth (LTB), belonged to the coliform group, with 51.6% being *Enterobacter agglomerans* and 23.9% being *E. coli*. Furthermore, Szabo and Todd (1997) stated in the MFHPB-27 document by the Health Protection Branch of the Government of Canada that the MTF method cannot detect or enumerate late lactose fermenters and anaerogenic *E. coli*. These problematic organisms are reported to comprise approximately 10.0% of all *E. coli* strains (Szabo & Todd, 1997). This approximation is confirmed by Fricker *et al.* (1997), who found 10.0% of *E. coli* strains from the Thames area could not ferment lactose. These organisms, which exhibit false-negative reactions, can seriously reduce the accuracy of the method due to the resultant under-estimation. Furthermore, the interference of non-coliform Gram negative bacteria (Edberg & Edberg, 1988) as well as heterotrophic bacteria present in excess of 500 cfu.mL<sup>-1</sup> (Evans *et al.*, 1981a) can severely impact on the sensitivity and specificity of the method.

Schiff *et al.* (1970) also reported that work done at the Colorado State University on the MTF method showed a high number of false-positive reactions in LST due to synergy between *Proteus* species and enterococci. Further validation has been recommended (Edberg & Edberg, 1988) to decrease the impact of false positives, which further increases the time until final results can be obtained. In addition, the production of gas in the temperature range of 44°-46°C is not an attribute that is possessed exclusively by *E. coli* (Leclerc *et al.*, 2001). Another factor in the MTF method which confounds the interpretation of results is the ability of a wide variety of organisms, other than *E. coli*, to hydrolyse MUG. The most important of these in the family *Enterobacteriaceae* are members of the genera *Shigella* and *Salmonella* (Iritani & Inzana, 1988).

These factors, which lead to false-positive reactions in the MTF method, can produce inaccurate results due to over estimation.

The potential inaccuracies which can occur in coliform and *E. coli* enumeration by the MTF method are the focus of this study. Consequently, the aim of the study is the phenotypic characterisation and identification of isolates from the four steps of the MTF and the confirmation of identification using a ribosomal protein spectral database. The identification of organisms is important for this study since the designation “atypical” or “typical” organism inextricably links the identity of the organism with its behaviour in media of the method.

**Note:** during the reporting of these results, the terms “aerogenic” and “anaerogenic” have been used for the description of isolates. In this context, this does not refer to the oxygen requirements of the organism, but the ability or inability to produce gas from lactose media.

### 5.3. MATERIALS AND METHODS

Coliforms and *E. coli* were isolated from MTF analyses of water collected in the Berg, Plankenburg, Eerste and Lourens Rivers in the Western Cape, South Africa. Isolates from the Berg River were obtained from MTF analyses done in the work for both Chapters 3 and 4, and isolates from the Plankenburg, Eerste and Lourens Rivers were obtained from MTF analyses done as part of Chapter 4. The same sampling locations as those used in Chapters 3 and 4 (where applicable) were used for all rivers. The MTF method used is outlined in the MFHPB-19 (Christensen *et al.*, 2002) document issued by the Government of Canada and is a version of the modified MTF as described by Evans *et al.* (1981a). A summary of the protocol is given in Table 5.1.

**Table 5.1.** MTF protocol used for the analysis of the river water samples

Step <sup>†</sup>	Temperature	Time	Observed reaction	Test for
LST broth	35.0 °C	24-48 h	Gas production & turbidity (growth)	Coliforms (presumptive)
BGLB broth	35.0 °C	24 h	Gas production & turbidity (growth)	Coliforms (confirmatory)
EC broth with MUG	44.5 °C	24 h	Gas production, turbidity (growth) & fluorescence with UV light (365 nm)	<i>E. coli</i>
L-EMB agar	35.0 °C	24 h	Dark colonies with iridescent green sheen	<i>E. coli</i> (completion/validation)

<sup>†</sup> All media obtained from Oxoid, Basingstoke, Hampshire, UK

For the purpose of this study, the terms “atypical” and “typical” reactions, and “atypical” and “typical” organisms, should be defined before discussion of the isolation methods used. The term “typical” reaction refers herein to any step of the method where the media exhibits all the

prescribed reactions as shown in Table 5.1 for the particular test organism or group of test organisms. An “atypical” reaction refers to any step of the method where some or all of prescribed reactions stipulated in Table 5.1 are absent.

The designation “atypical” or “typical” organism inextricably links the reaction caused by the organism to the organism’s identity. The term “typical” organism implies, in this context, only that the organism passes through the steps of the MTF method as expected. As an example *Enterobacter cloacae* behaving typically should only be detected up to the confirmatory step for coliforms, and a typical *E. coli* should progress through the method up to the production of typical metallic green colonies on L-EMB agar (Anon., 2005). In contrast, “atypical” describes an organism exhibiting an irregular reaction within the MTF method. For example, a member of the coliform group which progresses past the confirmatory step for coliforms to be counted as *E. coli* (over-estimation), and *E. coli* failing to be detected as such in the third broth step and on the confirmatory agar (under-estimation).

### 5.3.1. Isolation and purification

Isolates were from all broth steps as well as from the agar plates in the completed step, where both atypical and typical reactions were observed. Isolation was done from randomly selected tubes or agar plates, and the specific reaction and origin was recorded. Organisms were isolated from all broths by removing an inoculation loop from a well-mixed tube and streaking out on nutrient agar (NA, 16% (m/v) nutrient broth (Biolab, Merck, Wadeville, South Africa) with 12% (m/v) bacteriological agar (Biolab, Merck, Wadeville, South Africa)). Organisms were isolated from L-EMB agar by removing a single colony and streaking out on NA. All isolation procedures were done at the end of the incubation period (LST: 48h, BGLB: 24h, EC with MUG: 24h and L-EMB: 24h).

The isolates were re-streaked a minimum of three times on NA before streaking onto *E. coli*/Coliform Chromogenic Agar (Oxoid, Basingstoke, Hampshire, UK), henceforth referred to as “chromogenic agar”. The utilisation of the chromogenic dyes present in the agar leads to the production of purple colonies by *E. coli* (through targeting  $\beta$ -D-galactosidase and  $\beta$ -D-glucuronide) and pink colonies by coliforms (through targeting  $\beta$ -D-galactosidase) (Oxoid Limited, 2007). All non-coliform organisms appear as white colonies on the medium due to non-utilisation of the dyes. The colour reaction was noted for pure cultures. Although streaking on this medium was used as a purification step, the colour reaction of the isolate is also valuable as part of the phenotypic characterisation and will reported as such in the results section.

In most cases, only one isolate was obtained from an individual MTF tube, however, when two or three co-occurring isolates were found they were all individually purified and retained for further characterisation. These isolates are marked throughout the text with hyphenated numbers (e.g. 3-1 and 3-2).



### 5.3.2. Phenotypic characterisation and biochemical identification

Pure isolates were tested for the presence of catalase and cytochrome oxidase. All isolates were subjected to Gram staining for morphological examination under a light microscope (Nikon Eclipse E400, Nikon Corporation, Tokyo, Japan).

Each isolate was evaluated using the API 20E (API, bioMérieux SA, Marcy l'Étoile, Rhône, France) test strip. The miniaturised biochemical test strip, in addition to reporting 22 biochemical reactions of the isolate, also provided the data which were used to identify the organism using the *ApiWeb* software (bioMérieux SA, Marcy l'Étoile, Rhône, France).

Four American Type Culture Collection (ATCC) reference strains of *E. coli* (ATCC 11775, ATCC 4350, ATCC 10799 and ATCC 13135) were included in the phenotypic characterisation as positive controls. These strains were also subjected to the MTF method to ascertain whether they would produce “typical” reactions throughout the method.

### 5.3.3. Confirmation of identity

Since the identification of environmental isolates with systems or software based on clinical and laboratory strains can be troublesome, the identification of isolates based on the API strips and *ApiWeb* software was confirmed through the use of a MALDI Biotyper Microflex LT (Bruker Daltonik, GmbH, Bremen, Germany). This instrument is based on MALDI-TOF MS technology. The instrument has a database of nearly 4 000 organisms, characterised according to their ribosomal protein spectra. The highly conserved ribosomal proteins are different for each organism, and even for strains of the same organism, due to non-synonymous polymorphisms in housekeeping genes (Malorny *et al.*, 2011). These polymorphisms are expressed as slight variations in ribosomal proteins through the exchange of amino acids (Malorny *et al.*, 2011). Therefore, detection of these ribosomal proteins, which are conserved and thus fairly impervious to environmental adaptation, reduces the chance of incorrect identification due to adapted biochemical reactions in environmental isolates. When the spectrum of an unknown organism is analysed, the MALDI Biotyper compares its spectra with those in the library to identify it. The proteins that were tested for with the MALDI Biotyper are listed in Table 5.2 with their corresponding molecular weights.

**Table 5.2.** Ribosomal proteins tested for with the MALDI Biotyper Microflex LT

Ribosomal protein	Molecular weight (Da)
RL36	4365.3
RS32	5096.8
RS34	5381.4
RS33meth	6255.4
RL29	7274.5
RS19	10300.1
RNAse A [M+H] <sup>+</sup>	13683.2
Myoglobin [M+H] <sup>+</sup>	16952.3

The ribosomal proteins of each isolate were extracted using an ethanol/formic acid extraction method. Overnight colonies grown on NA were removed with sterile toothpicks and suspended in a mixture of GC grade water (Sigma-Aldrich, Kempton Park, South Africa) and absolute ethanol (Riedel de Haën, Sigma-Aldrich, Kempton Park, South Africa) in a ratio of 1:3 and a final volume of 1 200  $\mu$ L in an Eppendorf tube. After two centrifugation steps for 2 min at 14 000  $\times g$  in a Neofuge 13 benchtop centrifuge (Shanghai Lishen Scientific Equipment Company Limited, Shanghai, China), residual ethanol was removed with the aid of a micropipette. The pellet was air-dried for five minutes at ambient temperature before the addition of 50  $\mu$ L 70% (v/v) formic acid (Sigma-Aldrich, Kempton Park, South Africa), which was vigorously mixed with the dried pellet. An equal volume of pure acetonitrile (Sigma-Aldrich, Kempton Park, South Africa) was added to the reaction tube, which was then centrifuged for 2 min at 14 000  $\times g$ . The resulting supernatant was used for MALDI-TOF MS analysis.

A spot on the MSP 96 ground steel target plate (Bruker Daltonik, GmbH, Bremen, Germany) was covered with 1  $\mu$ L of supernatant from the protein extraction. After air-drying at ambient temperature the supernatant spot was immediately overlaid with 1  $\mu$ L of matrix solution, portioned  $\alpha$ -cyano-4-hydroxycinnamic acid (HCCA, Bruker Daltonik, GmbH, Bremen, Germany) dissolved in 250  $\mu$ L organic solvent prepared as a stock solution of 50% (v/v) pure acetonitrile (Sigma-Aldrich, Kempton Park, South Africa), 47.5% (v/v) GC grade water (Sigma-Aldrich, Kempton Park, South Africa) and 2.5% (v/v) trifluoroacetic acid (Sigma-Aldrich, Kempton Park, South Africa). The matrix was allowed to dry at ambient temperature before the target plate was inserted into the instrument for analysis. This was repeated for up to 96 protein extracts, since the target plate has 96 positions where different protein extracts can be spotted.

The instrument was calibrated once a day using the portioned Bacterial Test Standard (BTS, Bruker Daltonik, GmbH, Bremen, Germany), an extract of *E. coli* DH5alpha solubilised in 50  $\mu$ L of organic solvent from the stock solution and kept at -18°C. BTS was spotted onto the target plate and overlaid with matrix solution before being measured using the BTS\_FC.par method in the

flexControl software (Bruker). Baseline subtraction and smoothing was performed on the spectrum obtained, and the peak values were compared with the molecular mass of their corresponding proteins. When BTS peak values were all present and of acceptable molecular mass, they were assigned and saved as BTS\_process.FAMSMMethod in the flexControl program.

After insertion of the target plate into the calibrated instrument, the TOF device was evacuated to a vacuum of at least 1.19 Pa and the instrument's voltage was ramped to 20.0 kV and 16.9 kV for Ion Source 1 and 2, respectively. The analyses were done using the MBT\_FC.par method in the flexControl software (Bruker Daltonik, GmbH, Bremen, Germany) to control the laser. If low intensity peaks were obtained, the laser energy level was manually adjusted. The spectrum obtained from the isolate's protein extract was automatically compared with the peaks and intensities of spectra in the Bruker database using the MALDI Biotyper software, to identify the isolate (Cherkaoui *et al.*, 2010). The identification results provide the ten best matches in descending order and are each assigned a value by the MALDI Biotyper software which places it into one of four categories, based on the reliability of the identification as a log score between zero and three (Cherkaoui *et al.*, 2010). These categories are shown in Table 5.3.

**Table 5.3.** Result categories for MALDI Biotyper identification

Value	Level of identification
2.300-3.000	Highly probable species identification
2.000-2.299	Secure genus identification, probable species identification
1.700-1.999	Probable genus identification
0.000-1.699	Not reliable identification

If the value obtained for the first identification match of an isolate was less than 2.000, the ethanol/formic acid extraction procedure and MALDI-TOF MS analysis were repeated until an acceptable identification value was obtained. When the identification indicated possible contamination, extraction and analysis were performed in triplicate. If after this procedure no conclusive result was obtained, the results were recorded as is.

The four ATCC reference strains of *E. coli* were also used for identification confirmation with MALDI-TOF MS as positive controls.

## 5.4. RESULTS AND DISCUSSION

### 5.4.1. Isolation and purification

A collection of 111 organisms were isolated from the four steps of the MTF method: 3.6% of isolates from MTF on Eerste River water; 4.5% from Plankenburg River MTF analysis; 10.8% from

Lourens River MTF analysis; and 81.1% of isolates from MTF analysis on water from the Berg River. The distribution of these isolates across the four steps of the MTF method is shown in Table 5.4, as well as the number of isolates from atypical and typical reactions.

**Table 5.4.** Distribution of isolates and number of atypical/typical reactions in MTF steps

Step	Isolates (% of column total) <sup>a</sup>	Atypical reactions (% of row total) <sup>b</sup>	Typical reactions (% of row total) <sup>b</sup>
LST broth	35 (31.5)	33 (94.3)	2 (5.7)
BGLB broth	22 (19.8)	12 (54.5)	10 (45.5)
EC broth with MUG	41 (36.9)	14 (34.1)	27 (65.9)
L-EMB agar	13 (11.7)	10 (76.9)	3 (23.1)
<b>Total</b>	<b>111 (100.0)</b>	<b>69 (62.2)</b>	<b>42 (37.8)</b>

<sup>a</sup> numbers in brackets are percentages of the total number of isolates

<sup>b</sup> numbers in brackets are percentages of the number of isolates per broth/agar step

Atypical reactions for LST and BGLB broths were always taken as the presence of growth (indicated by turbidity) without gas production. Atypical reactions for EC broth with MUG were: turbidity and gas production with no fluorescence; turbidity with no gas production or fluorescence; turbidity and fluorescence with no gas production; and gas production with no turbidity or fluorescence. Atypical reactions for L-EMB agar were the appearance of any colony that was not dark with iridescent green sheen in reflected light.

#### 5.4.2. Phenotypic characterisation and identification of ATCC *E. coli* reference strains

The phenotypic characteristics and identification of the four *E. coli* reference strains, used as positive controls, are presented in Table 5.5.

**Table 5.5.** Summary of phenotypic characteristics and identification of four positive-control reference strains of *E. coli*

Strain	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
ATCC 11775	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.9
ATCC 4350	pink	+	-	-	Rods	<i>Escherichia coli</i> 1	99.9
ATCC 10799	pink	+	-	-	Short rods	<i>Escherichia coli</i> 1	99.9
ATCC 13135	purple	+	-	-	Short rods	<i>Escherichia coli</i> 1	99.9

The API identification results show that the ATCC *E. coli* strains are pure cultures with high identification percentages. Interestingly, two strains produced the purple colonies typical of *E. coli* on chromogenic agar; while two produced pink colonies associated with coliforms.

The four reference strains of *E. coli*, which served as positive controls, were first subjected to analysis by the MTF method. All four strains grew and produced gas in LST and BGLB broths; grew, produced gas and fluorescence in EC broth with MUG; and grew as metallic green colonies on L-EMB agar. Therefore, all four of the organisms were perfect typical MTF organisms.

#### 5.4.3. Phenotypic characterisation and identification of isolates from LST broth

The characteristics and biochemical identification of isolates from LST broth are given in Table 5.6. Since all organisms were isolated from a method which favours the growth of coliforms and *E. coli* and were catalase positive, oxidase negative and Gram negative rods (with one exception) the API 20E test strip was used, as this test is intended for the identification of enteric bacteria. Isolates with hyphenated numbers indicate more than one organism isolated from the reaction. Sizing of organisms as part of morphology was done visually.

**Table 5.6.** Some phenotypic characteristics and identifications of isolates from LST broth

Atypical reactions (MTF reaction: growth no gas)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
4	pink	+	-	-	Rods	<i>Aeromonas hydrophila</i>	85.9
5	pink	+	-	-	Rods	<i>Enterobacter sakazakii</i>	99.0
11-1	pink	+	-	-	Rods	<i>Enterobacter amnigenus</i>	67.8
11-2	white	+	-	-	Rods	<i>Morganella morganii</i>	99.9
12	pink	+	-	-	Very small rods	Unacceptable	
13-1	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.9
13-2	white	+	-	-	Very short rods	<i>Providencia alcalifaciens</i>	73.8
14	white	+	-	-	Small rods	<i>Providencia alcalifaciens</i>	71.7
15	pink	+	-	-	Rods	<i>Klebs. pneum.spp. ozaenae</i>	63.6
17	pink	+	-	-	Rods	Unacceptable	
21	white	+	-	(+)	Cocci	<i>Chromobact. violaceum</i>	99.5
26	NG	(+)	-	-	Rods	Unacceptable	
27	white	(+)	-	-	Rods	<i>Aeromonas salm. spp. salm.</i>	87.0
28	pink	+	-	-	Rods	<i>Klebs. pneum.spp. ozaenae</i>	88.5
30	white	+	-	-	Rods	Unacceptable	
35	pink	+	-	-	Rods	<i>Serratia marcescens</i>	97.4
39	white	+	-	-	Rods	<i>Hafnia alvei</i> 1	99.9
41	pink	+	-	-	Rods	<i>Enterobacter cloacae</i>	95.0
42	pink	+	-	-	Rods	Unacceptable	
43	pink	+	-	-	Rods	<i>Serratia rubidea</i>	92.2
44	white	+	-	-	Short rods	<i>Providencia rettgeri</i>	99.9

**Table 5.6 continued**

Table 5.6 continued

Atypical reactions (MTF reaction: growth no gas)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
46	pink	+	-	-	Rods	<i>Aeromonas</i> spp.	83.5
47	pink	+	-	-	Rods	<i>Citrobacter youngae</i>	99.8
48-1	pink	+	-	-	Short rods	<i>Escherichia coli</i> 1	99.5
48-2	white	+	-	-	Rods	<i>Serratia odorifera</i> 1	69.6
50	pink	+	-	-	Rods	<i>Chromobact. violaceum</i>	84.9
53	pink	+	-	-	Short rods	<i>Enterobacter aerogenes</i>	96.7
56	pink	+	-	-	Rods	<i>Enterobacter cloacae</i>	95.0
59	pink	+	-	-	Rods	<i>Serratia marcescens</i>	73.2
80	pink	+	-	-	Short rods	<i>Citrobacter freundii</i>	96.7
86	pink	(+)	-	-	Rods	<i>Pantoea</i> spp. 2	57.2
87	pink	+	-	-	Rods	<i>Enterobacter cloacae</i>	99.1
95	purple	+	-	-	Rods	<i>Enterobacter cloacae</i>	64.4
Typical reactions (MTF reaction: growth and gas)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
101	white	+	-	-	Very small rods	<i>Proteus vulgaris</i> group	99.9
109	white	+	-	-	Rods	<i>Providencia stuartii</i>	53.4

(+) - weak positive reaction

NG - no growth

It can be seen from the API identifications in Table 5.6 that a heterogeneous collection of organisms were obtained from LST broth. This was to be expected, since LST broth is the presumptive test for coliforms and facilitates the growth of genera belonging to the coliform group. It is, therefore, not surprising that 25 isolates (71.4%) belonged to genera which form part of the coliform group. Of all 35 isolates obtained from LST broth, five resulted in unacceptable profiles with API 20E while eight had identification percentages below 80%. This is possibly due to the fact that these isolates were obtained from an environmental source where they are expected to have undergone some adaptation, thereby limiting the ability of API 20E (which is based on clinical strains) to accurately identify them.

#### Atypical reactions in LST broth

Table 5.6 shows that 23 (92.0%) of the 25 isolates identified as coliforms from LST broth were from atypical reaction tubes which did not exhibit the production of gas. The coliforms which produced atypical reactions constituted 82.1% of the 28 identified (i.e. excluding unacceptable API profiles) organisms resulting in atypical reactions. This is lower than the results of LeChevallier *et al.* (1983), who found that 91.5% of anaerogenic organisms isolated from LTB belonged to the coliform group. However, the proportion of anaerogenic strains in a sample, especially an

environmental sample, is dependent on the nature of the sample. The work of LeChevallier *et al.* (1983) was done on chlorinated drinking water and lab-chlorinated samples, which could have increased the number of injured cells. These sublethally injured coliforms have been reported to have extended lag growth phases (Evans *et al.*, 1981a), and may not have recovered sufficiently and repaired their DNA (Edberg & Edberg, 1988) to produce gas. This higher prevalence of sublethally injured coliforms may be responsible for the discrepancy in the proportion of anaerogenic coliforms which was observed between the results of LeChevallier *et al.* (1983) and in this study.

Anaerogenesis could be attributable to the absence of either lactose permease (Fricker *et al.*, 1997) or  $\beta$ -D-galactosidase, which catalyses the hydrolysis of lactose to glucose and galactose (Manafi *et al.*, 1991). In addition, even if  $\beta$ -D-galactosidase is present in the cell it will not necessarily be expressed in an inhibitory medium (Niemela *et al.*, 2003). The failure of these coliforms to produce gas from lactose could also be due to the absence of some element in the hydrogenlyase reaction, tasked with the production of CO<sub>2</sub> and H<sub>2</sub> gas from formic acid (Gest & Peck, 1955). Formic acid is an intermediate in the degradation of pyruvate (Gest & Peck, 1955; Leonhartsberger *et al.*, 2002) which, in turn, is an intermediate in the degradation of lactose (as glucose) from the glycolytic cycle (Edberg *et al.*, 1988). The hydrogenlyase reaction is a multi-enzyme system consisting of formic dehydrogenase, an electron carrier and hydrogenase. If any of these factors are missing from the system, gas will not be produced from formic acid (Gest & Peck, 1955; Bagramyan & Trchounian, 2003).

It is also possible that no gas was produced due to coliform suppression by autochthonous heterotrophic bacteria present (Evans *et al.*, 1981a; Edberg & Edberg, 1988) in the river water. This is attributable to the competition for nutrients, as well as the secretion of bacteriocins (Edberg & Edberg, 1988). Finally, injured cells may also fail to produce gas due to an increased lag time (Evans *et al.*, 1981a) or due to an increased sensitivity for surface active agents (Niemela *et al.*, 2003) such as sodium lauryl sulphate, a constituent of LST broth.

The reason for the lack of gas production in 92.0% of coliforms isolated from LST broth, although admittedly important, stands secondary to the implications of their failure to ferment lactose. Isolation was done at the end of 48 hours of incubation, specifically designed to benefit slow lactose fermenters and environmental strains which grow at a slower rate (McFeters *et al.*, 1997), meaning isolates would have no additional time to produce gas. Since LST broth is the presumptive test for coliforms, 92.0% of the coliform isolates, including two isolates of *E. coli*, would not have been enumerated as presumptive coliforms and would, according to the method, not have been transferred to any subsequent enumeration steps. When considering these factors, the presence of these isolates would result in a considerable under-estimation of the amount of presumptive coliforms, and, worse yet, result in under-estimation during further steps of the MTF method. These organisms, isolated from atypical reactions, also fit the definition of the study for “atypical organisms”.



The five isolates which did not fall into genera within the coliform group were all isolated from LST broth exhibiting atypical reactions. These isolates were 4, 21, 27, 46 and 50; and all belong either to the genus *Aeromonas* or *Chromobacterium*. There exists some doubt, however, regarding the identities of isolates 21 and 50. The Gram reactions, morphology and growth on chromogenic agar of the isolates differed even though both were identified as *Chromobacterium violaceum*. Isolate 21 exhibited discrepant morphology for the species, although the morphology of *C. violaceum* is sometimes described as coccobacilli, and also an ambiguous Gram reaction, but produced a higher identification percentage with the API. The presence of *Aeromonas* among non-detected isolates is encouraging, as literature reports that *Aeromonas* spp. possess  $\beta$ -D-galactosidase (Geissler *et al.*, 2000) and can ferment lactose, and consequently cause false-positives (Edberg & Edberg, 1988). The suppression of these potential false-positive aeromonads can be attributed to the inhibitory compounds, such as the sodium lauryl sulphate in LST broth. These five isolates would not have been enumerated as presumptive coliforms and would not have resulted in over-estimation and, although isolated from atypical reactions, are “typical organisms” according to the definition for the purpose of the study.

#### Typical reactions in LST broth

The two organisms isolated from typical reaction tubes were found to belong to the genera *Proteus* and *Providencia*. These are genera within the coliform grouping (Leclerc *et al.*, 2001), and since these isolates produced gas in LST broth they would be enumerated as presumptive coliforms. The organisms, isolated from typical reactions, are also “typical organisms” as per the definition of the study.

#### Unacceptable profiles

No conclusions can be drawn from the five isolates with unacceptable API identifications from the data presented in Table 5.6.

#### Reactions on chromogenic agar

The chromogenic agar results shown in Table 5.6 showed some unexpected reactions. Three non-coliform isolates (4, 46 and 50) exhibited pink colonies, typical of coliforms (Oxoid Limited, 2007), on the chromogenic agar. These anomalies could possibly be a result of these organisms possessing enzyme systems that can utilise the dyes and cause these colour changes, or due to misidentification with API since none of these isolates had very high identification percentages.

Eight coliform isolates (11-2, 13-2, 14, 39, 44, 48-2, 101 and 109) also produced white colonies on chromogenic agar, which is typical of non-coliform growth. It is possible that these isolates may not possess the  $\beta$ -D-galactosidase necessary for the production of the characteristic pink colour, but, as with the previous case, no comment can be made on the accuracy of

chromogenic agar. If the results of the chromogenic agar prove to be correct, the number of non-coliform “typical organisms” will increase from five to 13 or 14, depending on the identity of isolate 30.

Isolate 48-1, identified as *E. coli*, produced pink colonies on chromogenic agar, and not the purple colonies typical of *E. coli*. This could also be ascribed to the lack of  $\beta$ -D-glucuronidase, since the likelihood of misidentification is low (%ID = 99.5). In addition, isolate 95, identified as *Enterobacter cloacae*, produced purple colonies which, according to the manufacturer, should represent *E. coli*. The discrepancy is not of great concern here, since LST tests for the presumptive coliform group, which includes both organisms. If the API identifications are correct, however, doubts regarding the ability of chromogenic agar to discriminate between *E. coli* and coliforms would arise.

#### Co-occurring isolates

In three of the atypical reactions, two organisms were isolated per reaction. This low proportion of co-occurrence events are probably due to inhibition of one isolate over another on the chromogenic medium in most cases, and should not be seen as the *in situ* incidence of co-occurrence in MTF tubes which is accepted to be high. This is especially true for LST broth, where the bacterial population is still very heterogeneous. Isolates 11-1 and 11-2 were members of the genera *Enterobacter* and *Morganella*, respectively. Isolate 13-1 was identified as *E. coli*, while its co-occurring isolate 13-2 was identified as *Providencia alcalifaciens*. Similarly, isolate 48-1 was identified as *E. coli*, and 48-2 as *Serratia*. All these organisms fall within the coliform group. It is perplexing, therefore, that no gas was produced in the LST broth when these co-occurring isolates were found. Had it been a case of one organism inhibiting the growth of the other, the dominating organism would still have produced gas. Consequently, these organisms must either both be anaerogenic coliforms, mutually inhibiting another's gas production, using the metabolites produced by the other organism, or have been identified incorrectly by the API system.

#### 5.4.4. Phenotypic characterisation and identification of isolates from BGLB broth

The phenotypic characteristics and identification of organisms isolated from BGLB broth are presented in Table 5.7. Since all organisms were catalase positive, oxidase negative and Gram negative rods, the API 20E test strip was used. Isolates with hyphenated numbers indicate more than one organism isolated from the reaction. Sizing of organisms as part of morphology was done visually.

**Table 5.7.** Some phenotypic characteristics and identifications of isolates from BGLB broth

Atypical reactions (MTF reaction: growth no gas)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
22	white	+	-	-	Strings of rods	<i>Morganella morganii</i>	99.9
23	white	+	-	-	Short rods	<i>Providencia rettgeri</i>	99.3
24-1	pink	+	-	-	Very small rods	Unacceptable	
24-2	white	+	-	-	Rods	<i>Morganella morganii</i>	99.9
25	pink	+	-	-	Short rods	<i>Enterobacter cloacae</i>	95.0
29	pink	(+)	-	-	Rods	<i>Klebsiella oxytoca</i>	94.8
34	pink	+	-	-	Rods	<i>Enterobacter cloacae</i>	95.0
38	pink	+	-	-	Very short rods	<i>Klebsiella oxytoca</i>	97.9
40	pink	+	-	-	Very short rods	<i>Klebsiella oxytoca</i>	97.9
51	pink	(+)	-	-	Rods	<i>Klebsiella oxytoca</i>	94.8
75	pink	(+)	-	-	Rods	<i>Enterobacter aerogenes</i>	96.7
92	white	+	-	-	Rods	<i>Pantoea</i> spp. 4	68.6
Typical reactions (MTF reaction: growth and gas)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
98	white	+	-	-	Rods	<i>Proteus mirabilis</i>	99.0
99	white	+	-	-	Rods	<i>Proteus vulgaris</i> group	98.8
100	white	+	-	-	Rods	<i>Proteus mirabilis</i>	99.9
102	white	+	-	-	Rods	<i>Proteus vulgaris</i> group	99.9
103	white	+	-	-	Rods	<i>Proteus mirabilis</i>	99.0
104	white	+	-	-	Long, thin rods	<i>Providencia stuartii</i>	98.9
105	white	+	-	-	Rods	<i>Proteus mirabilis</i>	99.9
106	white	+	-	-	Small rods	<i>Proteus mirabilis</i>	99.9
107	white	+	-	-	Rods	<i>Proteus vulgaris</i> group	99.9
108	pink	+	-	-	Short rode	Unacceptable	

(+) - weak positive reaction

The variation in API identifications found in Table 5.7 indicates that there is less heterogeneity in isolates from BGLB than those from LST broth. This was to be expected, since BGLB broth presents additional selective hurdles for growth, through the inclusion of bile and brilliant green, to determine the confirmed coliform count. All the isolates which could be identified by API (90.9%) belonged to genera of the coliform group. This is not surprising, since organisms detected in BGLB broth have been transferred from LST broth, where they were classified as presumptive coliforms. It is possible, however, that additional “passenger” non-coliforms can be transferred with a coliform from a tube with gas production. This is not immediately troublesome, since a tube with gas production by a coliform would have been counted as such irrespective of the additional presence of non-coliforms. It can, however, become problematic if these “passenger”

organisms have an inhibitory function on the test organism(s) in the later steps of the MTF method through their production of metabolites or preferential utilisation of nutrients.

#### Atypical reactions in BGLB broth

Of the organisms isolated from BGLB broth, 11 (55.0%) were isolated from BGLB broth with atypical reactions. Therefore, the isolates would not have been enumerated as confirmed coliforms, despite their identifications showing that they all belong to genera within the coliform group. The majority of these “atypical organisms” belong to the genera *Klebsiella* (36.4%), *Enterobacter* (27.3%) or *Morganella* (18.2%). Thus, 55.0% of identified coliforms from BGLB broth would not have been enumerated. This would have lead to an under-estimation of confirmed coliforms *in situ* of potentially more than half of the confirmed coliforms. The considerable proportion of anaerogenic coliforms found in BGLB broth compares well with reports (Ruchhoft, 1935; Ruchhoft & Norton, 1935) citing the failure of subcultured coliforms to grow in BGLB after they had been transferred from a lactose-containing medium (such as LST broth). Evans *et al.* (1981a) also raised doubts regarding the appropriateness of using BGLB broth as confirmatory test in chlorinated and untreated surface water.

#### Typical reactions in BGLB broth

The identified organisms originating from typical BGLB reactions all belonged to either *Proteus* or, in once instance, *Providencia*. Since both these genera fall within the coliform group, and BGLB enumerates confirmed coliforms, all of these isolates would have been correctly enumerated as coliforms. It is interesting to observe that the isolates from typical reactions in LST also belonged to *Proteus* and *Providencia*. It appears from this observation as though LST and BGLB broths are ideally suited for the growth of organisms from these two genera, and that they dominate the population of gas-producing organisms in these two steps and thereby “carry” faecal coliforms, including *E. coli*, to the steps where they are to be detected and enumerated.

#### Unacceptable profiles

The implications of the two organisms with unacceptable API identifications were not included in this discussion, since no conclusions regarding their impact on this step can be made without knowledge of their identities. However, their production of pink colonies on chromogenic agar alludes to the likelihood that they are coliforms.

#### Reactions on chromogenic agar

Some of the chromogenic results in Table 5.7 are, once again, somewhat puzzling. Of the 20 identified coliforms, 13 (65.0%) produced white colonies, typical of non-coliforms, on chromogenic agar and belonged to the genera *Proteus*, *Providencia*, *Morganella* and *Pantoea*. This is probably

due to a lack of functional  $\beta$ -D-galactosidase, required to cause the characteristic pink colonies in these organisms. Alternatively, the likelihood exists that these organisms were incorrectly identified due to atypical biochemical reactions with the API 20E test. If this is the case and they are not coliforms, the isolates from typical reaction tubes would lead to over-estimation while the degree of under-estimation from atypical tubes would decrease. However, when referring back to coliforms from LST broth which resulted in white colonies on chromogenic agar, it is interesting to note that they belong to the genera *Providencia*, *Proteus*, *Morganella*, *Hafnia* and *Serratia*. It is unlikely that such a similarity is coincidental, and the theory of lacking enzyme systems in these genera becomes more plausible than that of incorrect identification.

#### Co-occurring isolates

In one atypical reaction from BGLB broth, two organisms were found to be co-occurring. Isolate 24-1 had an unacceptable identification with API, but isolate 24-2 was identified as *Morganella morganii*. The latter falls in the coliform group and is expected to produce gas. The former, however, although suspected of being a coliform, could be a heterotrophic bacteria inhibiting gas production by *Morganella*. This would agree well with reports by Evans *et al.* (1981a), who observed the suppression of coliforms by heterotrophs. If isolate 24-1 is a coliform, the lack of gas production could be attributable to anaerogenesis of both isolates, mutual inhibition of the production of gas, utilisation of metabolites produced by the other organism, or due to misidentification by API.

#### 5.4.5. Phenotypic characterisation and identification of isolates from EC broth with MUG

The phenotypic characteristics and biochemical identification of organisms isolated from EC broth with MUG are presented in Table 5.8. Since all organisms were catalase positive, oxidase negative and Gram negative rods, the API 20E test strip was used. Isolates with hyphenated numbers indicate more than one organism isolated from the reaction. Sizing of organisms as part of morphology was done visually.

**Table 5.8.** Some phenotypic characteristics and identifications of isolates from EC broth with MUG

Atypical reactions (MTF reaction: growth, fluorescence, no gas)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
6	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.4
7	purple	+	-	-	Short rods	<i>Klebs. pneum. spp. pneum</i>	97.7
69	pink	+	-	-	Short rods	Unacceptable	
70	pink	+	-	-	Short rods	Unacceptable	
Atypical reactions (MTF reaction: growth, no fluorescence, no gas)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
8	pink	+	-	-	Rods	<i>Escherichia coli</i> 1	99.4
37	pink	+	-	-	Short rods	Unacceptable	
72	pink	+	-	-	Rods	Unacceptable	
94	pink	+	-	-	Rods	<i>Citrobacter freundii</i>	99.3
97	pink	+	-	-	Short rods	<i>Enterobacter asburiae</i>	45.5
Atypical reactions (MTF reaction: growth, gas, no fluorescence)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
18	pink	(+)	-	-	Rods	<i>Escherichia coli</i> 1	99.5
19	pink	+	-	-	Small rods	<i>Escherichia coli</i> 1	99.9
20	pink	+	-	-	Cocco-bacilli	<i>Klebsiella oxytoca</i>	96.2
36	pink	+	-	-	Rods	Unacceptable	
Atypical reaction (MTF reaction: gas, no growth, no fluorescence)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
9	pink	(+)	-	-	Rods	<i>Klebs. pneum. spp. pneum</i>	97.7
Typical reactions (MTF reaction: gas, growth, fluorescence)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
54	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.8
55	purple	+	-	-	Short rods	<i>Escherichia coli</i> 1	99.8
57-1	purple	+	-	-	Short rods	<i>Escherichia coli</i> 1	99.5
57-2	pink	+	-	-	Small rods	<i>Enterobacter cloacae</i>	59.2
60	purple	+	-	-	Short rods	<i>Escherichia coli</i> 1	99.5
61	purple	+	-	-	Rods	<i>Kluyvera</i> spp.	98.3
62	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.9
63	purple	+	-	-	Short rods	<i>Escherichia coli</i> 1	50.0
64-1	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.5
64-2	pink	+	-	-	Rods	<i>Enterobacter sakazakii</i>	98.4

**Table 5.8 continued**

Table 5.8 continued

Typical reactions (MTF reaction: gas, growth, fluorescence)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
65	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.5
66	purple	+	-	-	Short rods	<i>Escherichia coli</i> 1	99.8
67	pink	+	-	-	Rods	<i>Escherichia coli</i> 1	99.8
68	purple	+	-	-	Short rods	<i>Escherichia coli</i> 1	99.5
71*	pink	+	-	-	Short rods	Unacceptable	
73-1*	purple	+	-	-	Small rods	<i>Escherichia coli</i> 1	63.8
73-2*	pink	+	-	-	Rods	<i>Escherichia coli</i> 1	98.6
73-3*	white	+	-	-	Short rods	Unacceptable	
74	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.5
79	purple	+	-	-	Short rods	<i>Escherichia coli</i> 1	99.9
82	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.8
84-1	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.8
84-2	pink	+	-	-	Rods	<i>Escherichia coli</i> 1	99.9
85	purple	+	-	-	Short rods	<i>Escherichia coli</i> 1	99.9
88	pink	+	-	-	Short rods	Unacceptable	
89	pink	+	-	-	Small rods	<i>Serratia marcescens</i>	99.8
90	pink	+	-	-	Short rods	Unacceptable	

(+): weak positive reaction

GR: growth (turbidity), G: gas production, F: fluorescence

\*: organisms with weak gas reactions

The API identifications presented in Table 5.8 indicate that the group of organisms isolated from EC broth with MUG shows decreased heterogeneity in isolate identity when compared to BGLB and, in particular, LST broths. This is most apparent in isolates from typical reaction tubes. The latter observation is to be expected, since EC broth with MUG challenges confirmed coliforms, transferred from BGLB broth, with additional selective hurdles through the inclusion of bile and MUG. Additionally, the elevated incubation temperature of 44.5°C was designed to select only thermotolerant coliforms. The relative homogeneity in the identity of isolates from atypical reaction tubes can be attributed to the various selective hurdles that the organisms already overcame in LST and BLGB broth, to be classified as confirmed coliforms. Therefore, it is not surprising that *E. coli* constitutes the majority (23 isolates or 71.9%) of the 32 identified isolates from EC broth with MUG. Despite the high prevalence of *E. coli* isolates from this step, the abundance of non-*E. coli* organisms (28.1%) is higher than might be expected from a medium which was designed to enumerate *E. coli* and exclude all other organisms. Nine isolates obtained from EC broth with MUG could not be identified by API 20E, presumably due to adaptations which altered their biochemical profiles.



### Atypical reactions in EC broth with MUG

Isolates originating from atypical reactions were isolated from tubes with one of four permutations, judged based on the absence or presence of growth, gas and fluorescence in the medium.

#### *Growth, fluorescence and no gas*

Two organisms (6 and 7) isolated from tubes exhibiting growth, fluorescence and no gas production were identified as *E. coli* and *K. pneumoniae* spp. *pneumoniae*. The other two (69 and 70) isolates from tubes with this reaction had unacceptable identifications with API. Their chromogenic reactions, however, indicated that these organisms are coliforms. It is possible that an ingredient in EC broth, which is intended to enumerate thermotolerant coliforms (and specifically *E. coli*, with the addition of MUG), inhibited the gas production by isolates 69 and 70. Alternatively, or perhaps additionally, it is possible that the elevated temperature inhibited their growth and, consequently, their fermentation of lactose in the medium.

Isolates 6 and 7 both resort under the thermotolerant coliform group as described by Leclerc *et al.* (2001) and would be expected to grow and produce gas at 44.5°C. Therefore, the failure of these organisms to produce gas in the medium suggests that they are anaerogenic strains of *E. coli* and *K. pneumoniae*. Anaerogenesis and failure to grow at 44.5°C by some strains of *E. coli* is not impossible (Leclerc *et al.*, 2001), and is reported by some workers to be more prevalent than the lack of  $\beta$ -D-glucuronidase activity (Niemela *et al.*, 2003; Wohlsen *et al.*, 2008). *K. pneumoniae* would have, correctly, not been enumerated *in situ*. Isolate 6, however, would not have been enumerated and would have resulted in an under-estimation of the number of *E. coli* present in the sample. An additional concern is the fluorescence observed in tubes containing isolates 7, 69 and 70. Reports have indicated that  $\beta$ -D-glucuronidase activity is widespread among bacterial genera, although limited to a few strains within a genus (Sercu *et al.*, 2011). Some strains of *K. pneumoniae* have been identified by Chang *et al.* (1989) as possessing  $\beta$ -D-glucuronidase. Although not surprising, the production of fluorescence by these three organisms could be problematic if they were to occur together with a gas-producing organism not belonging to *E. coli*. The synergistic production of a false-positive tube could result in an over-estimation of the number of *E. coli* present in the sample.

#### *Growth, no fluorescence and no gas*

Five organisms (8, 37, 72, 94 and 97) were isolated from atypical reaction tubes exhibiting growth, but no fluorescence or gas production. Three of these isolates were identified as *E. coli*, *C. freundii* and *Entb. asburiae*. The remaining two isolates gave unacceptable profiles, but, once again, their production of pink colonies on chromogenic agar pointed to the likelihood of them being coliforms. The four isolates not identified as *E. coli* are not problematic in this situation, as

they were only able to grow and cause turbidity in the tube, but could not produce gas. Therefore, they would not have been counted as *E. coli* and would not have resulted in an over-estimation of *E. coli*. It is interesting to note, however, that some strains of *C. freundii* are grouped with the thermotolerant coliforms (Leclerc *et al.*, 2001). If MUG had been omitted from the assay, and the test was for thermotolerant coliforms, isolate 94 would not have been enumerated as a thermotolerant coliform due to its lack of gas production. From the atypical reaction from which it was isolated, it would appear that isolate 8, which was identified as *E. coli*, is an anaerogenic and  $\beta$ -D-glucuronidase negative strain of *E. coli*. If it was encountered *in situ*, it would not have been counted and would have resulted in under-estimation.

#### *Growth, gas and no fluorescence*

Four organisms (18, 19, 20 and 36) were isolated from atypical reaction tubes exhibiting growth and gas, but no fluorescence. Two of these were identified as *E. coli*, one as *K. oxytoca*, and one resulted in an unacceptable API profile. The organism with an unacceptable profile once again produced pink colonies on chromogenic agar, and it was suspected to be a coliform.

The two isolates that were identified as *E. coli* are presumably  $\beta$ -D-glucuronidase negative strains, since this would explain their inability to hydrolyse MUG. *E. coli* strains of this nature represent approximately 4-6% (Manafi *et al.*, 1991; Manafi, 2000) of the species. These isolates could be problematic if MUG is included in the assay, since they would not be counted as *E. coli*. With the omission of MUG, however, the assay tests for thermotolerant coliforms, and these two isolates would have been counted as such due to their growth and gas production.

The isolate of *K. oxytoca* poses no threat to the accuracy of enumeration of the test, since this organism is a thermotolerant coliform (Leclerc *et al.*, 2001). It is, therefore, not expected to produce a fluorescent reaction with MUG, although such reactions have been reported (Manafi, 2000). If MUG was omitted from the assay, the isolate would have been correctly counted as a thermotolerant coliform. There is, however, one potential problem with this organism: if it is to co-occur in a EC broth plus MUG tube with an organism like isolate 69, which produced growth, fluorescence and no gas; these two organisms could synergistically be responsible for a false-positive reaction that would be counted as *E. coli*.

The isolate (36) which produced an unacceptable profile with API, but is very likely to be a coliform, poses a similar risk as that of the *K. oxytoca* isolate: if it is a thermotolerant coliform it would be correctly enumerated in the absence of MUG, but if it occurred with an organism such as isolate 69 it could lead to a synergistic false-positive *E. coli* enumeration in the presence of MUG.

#### *Gas, no visible growth and no fluorescence*

One atypical reaction manifesting as gas with no visible growth or fluorescence was also encountered. Since the fermentation of lactose without the presence or growth of an organism is

highly unlikely in this scenario, it must be concluded that some growth must have occurred. This growth was, however, not visually observable and explains the confusing description of the reaction. It should also be noted that this reaction was by no means typical: the absence of any turbidity or biomass in the tube would immediately have disqualified it for enumeration *in situ*. The isolate (9) responsible for this atypical reaction was identified as *K. pneumoniae* spp. *pneumoniae*. This organism was clearly incapable of hydrolysing MUG or growing effectively in the medium, and would not be counted with or without the addition of MUG to EC broth. This would result in under-estimation with the thermotolerant coliform assay, since *K. pneumoniae* is grouped with the thermotolerant coliforms according to Leclerc *et al.* (2001). The organism could also be detrimental to the accuracy of results if it occurs with an organism such as isolate 69, since the combined reactions would result in a false-positive result for *E. coli*.

#### Typical reactions in EC broth with MUG

*E. coli* comprised the majority (70.4%) of isolates from typical reactions in EC broth with MUG (n=27). This was to be expected, since EC broth with MUG in conjunction with incubation at 44.5°C was designed to exclusively enumerate *E. coli* (Neogen Corporation, 2010; Burlage, 2011). However, four other isolates were identified as *Entb. cloacae*, *Kluyvera* spp., *Entb. sakazakii* and *S. marcescens*. In addition, four isolates could not be identified with API and yielded unacceptable profiles. Their reactions on chromogenic agar were varying: three (71, 88 and 90) produced pink colonies typical of coliforms, and one (73-3) produced white colonies typical of non-coliforms. Since all organisms inoculated into EC broth with MUG had to pass the confirmed coliform test in BGLB, and should at least be coliforms, the possibility exists that the isolate producing white colonies could belong to one of the previously discussed genera within the coliform grouping which have strains which produce white colonies on chromogenic agar.

The *E. coli* isolates from typical reactions would not lead to under-estimation, since they would all have been enumerated as *E. coli*. If MUG was omitted from the assay, these organisms would have produced growth and gas and would consequently have been identified as thermotolerant coliforms. Therefore, the reactions of these organisms would have resulted in their correct enumeration irrespective of the addition or omission of MUG from the assay.

The isolates identified as *Kluyvera* spp. and *S. marcescens* hold negative implications for the accuracy of enumeration, since these organisms produced an altogether typical EC broth with MUG reaction and would be counted *in situ* as *E. coli*, resulting in the over-estimation of *E. coli*.

Caution should be exercised when interpreting and discussing the implications of isolates 57-2, 64-2 and 73-3, since these organisms were co-isolated with *E. coli*. These organisms will be discussed under the co-occurring isolates section.

### Reactions on chromogenic agar

The reactions on chromogenic agar for isolates from EC broth with MUG have been discussed in previous sections to aid in the interpretation of other results. It is important to note however that of the 23 *E. coli* organisms isolated from both typical and atypical reactions, six (26.1%) *E. coli* isolates produced pink colonies on chromogenic agar, and not the purple colonies typical of *E. coli*. This is possibly due to a missing, inactive or non-functional  $\beta$ -D-glucuronidase enzyme system responsible for the colour reaction. Although there is always the possibility of incorrect identification due to discrepant reactions with API 20E, it is unlikely that 26.1% of isolates, were incorrectly identified. Additionally, the same phenomenon was observed with an *E. coli* (48-1) isolated from LST broth.

The only other discrepancies in Table 5.7 are the purple colonies on chromogenic agar produced by isolate 7, *K. pneumoniae* spp. *pneumoniae*, and 61, *Kluyvera* spp. In this case, the theory of a lacking enzyme system cannot explain this observation. Instead, the observed production of purple colonies on chromogenic agar is indicative of the presence of  $\beta$ -D-glucuronidase in isolates 7 and 61.

### Co-occurring isolates

The only co-occurring isolates found in EC broth with MUG were isolated from typical reactions. Isolates 57-2, 64-2 and 73-3 were all isolated from tubes where *E. coli* was also present. Therefore, it cannot be concluded that these isolates resulted in false-positive reactions, since the typical reactions cannot necessarily be attributed to them. However, the typical reactions cannot necessarily be attributed to the co-occurring *E. coli* either, but the likelihood of this being the case is much larger. If these organisms were isolated by chance and were not involved in the production of the typical reactions, there would be no implication for the method since *E. coli* was present and enumerated. If these isolates do, however, contribute to the production of the typical reactions, they can impact on the accuracy of the method. If they are solely responsible for the typical reactions, they would be counted as *E. coli*, which would result in over-estimation. If they are partially responsible for the typical reactions and contribute synergistically with a partial typical reaction from *E. coli* to a whole typical reaction, they could assist the enumeration of an "atypical" *E. coli* which would otherwise not have been counted. Although this synergy would increase the accuracy of enumeration of *E. coli*, its occurrence in the MTF is a random event and cannot be relied on to consistently aid in the enumeration of "atypical" *E. coli*. The uncertainty raised by the co-occurring isolates will be addressed in a later chapter of this dissertation (Chapter 8), where individual isolates will be tested to determine their contribution to the mixed MTF reaction.

#### 5.4.6. Phenotypic characterisation and identification of isolates from L-EMB agar

Some phenotypic characteristics and identification of organisms isolated from L-EMB agar are presented in Table 5.9. Since all organisms were catalase positive, oxidase negative and Gram negative rods, the API 20E test strip was used. Isolates with dashed numbers indicate more than one organism isolated from the colony. Sizing of organisms as part of morphology was done visually.

**Table 5.9.** Some phenotypic characteristics and identifications of isolates from L-EMB agar

Atypical reactions (MTF reaction: dark purple colonies)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
1	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	89.7
45	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.1
Atypical reactions (MTF reaction: mucoid purple colonies)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
2	pink	+	-	-	Rods	<i>Klebs. pneum. spp. pneum.</i>	97.7
81-1	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.8
81-2	pink	+	-	-	Short rods	<i>Pantoea</i> spp. 2	63.7
93	pink	+	-	-	Rods	<i>Enterobacter cloacae</i>	99.1
Atypical reactions (MTF reaction: mucoid pink colonies)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
3-1	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.9
3-2	pink	+	-	-	Short rods	Unacceptable	
16	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.9
32	pink	+	-	-	Rods	<i>Pseudomonas aeruginosa</i>	99.9
Typical reactions (MTF reaction: metallic green colonies)							
Isolate	Chromo-genic	Cata-lase	Oxi-dase	Gram	Morphology	API identification	%ID
31	purple	+	-	-	Rods	<i>Escherichia coli</i> 1	99.8
49	pink	+	-	-	Short rods	Unacceptable	
52	purple	+	-	-	Short rods	<i>Escherichia coli</i> 1	99.9

The variation of isolate species obtained from L-EMB agar showed that the isolates were surprisingly heterogeneous, when considering that this final step is the validation of *E. coli* enumeration by EC broth with MUG. *E. coli* organisms constituted 53.8% of all isolates. However, it should be remembered that this proportion was obtained for a group of isolates where a large number of isolations were done from atypical reactions. Therefore, this proportion is not indicative of the relative abundance of *E. coli* on L-EMB agar *in situ*.

### Atypical reactions on L-EMB agar

Half (5 out of 10) of the isolates obtained from atypical growth on L-EMB agar were identified as *E. coli*, while the remaining half was represented by organisms from the genera *Klebsiella*, *Pseudomonas*, *Pantoea* and *Enterobacter*. It should be kept in mind that the presence of *E. coli* among atypical growth reactions, although troublesome, shows that the preceding steps of the MTF method lead to correct enumeration of *E. coli*. Two of the *E. coli* isolates (1 and 45) grew as dark colonies, typical on L-EMB, but without the typical iridescent green sheen. It is possible that these organisms are only lacking in the mechanism for producing the green sheen, which is associated with acidic conditions (Schleinitz, 2005) brought about by the fermentation of lactose in the medium.

More troubling, however, is the presence of thermotolerant coliforms (*K. pneumoniae* spp. *pneumoniae* and *Entb. cloacae*), coliforms (*Pantoea* spp. 2) and even non-coliforms (*Pseudomonas aeruginosa*) on this agar. In addition, an isolate with no acceptable identification, which produced pink colonies on chromogenic agar, was also present in one instance. Although these organisms were found in atypical reactions, their implications are concerning: they would have had to pass through three broth steps and various selective hurdles and were consequently enumerated as presumptive and confirmed coliforms, as well as *E. coli*, before arriving on L-EMB agar. This observation indicates that these organisms would have, during an *in situ* MTF analysis, lead to an over-estimation of *E. coli* numbers in EC broth with MUG. Fortunately, if these organisms are detected on L-EMB agar as not belonging to *E. coli*, the EC broth MUG enumeration of *E. coli* can be amended to exclude the false-positive tubes.

It is, of course, also possible than they were co-occurring in the preceding tubes with true test organisms, and were transferred with the latter. This conclusion explains the presence of isolates 3-2 and 81-2, which were isolated from co-occurrence with *E. coli*.

### Typical reactions on L-EMB agar

Two of the three isolates from typical growth on L-EMB agar were identified as *E. coli*. This was an expected result, and holds no negative implications for the method. The third isolate, however, was unidentifiable by API. Its reaction on chromogenic agar was the production of pink colonies, which indicates the possibility that it could be a coliform. If this is the case, it is problematic to find a coliform on the validation medium, since that means that it has been incorrectly enumerated as *E. coli* in the previous step of the MTF method. However, the possibility of this isolate being an *E. coli* should not be discounted, since various *E. coli* isolates from LST and EC broth with MUG were found to produce pink colonies on chromogenic agar.

### Reactions on chromogenic agar

Apart from the unacceptable profiles, where the detection efficacy of chromogenic agar cannot be interpreted, most of the other organisms reacted on the agar as would be expected. All *E. coli* isolates exhibited typical purple colonies, and the coliforms produced typical pink colonies. The only anomaly was the production of pink colonies by *Pseudomonas aeruginosa*, which suggests the possibility of misidentification.

### Co-occurring isolates

The only cases of co-occurrence were found in isolates 3-1 and 3-2, and isolates 81-1 and 81-2. Isolate 3-2 is a strain of *K. pneumoniae* which was isolated from a mixed culture with *E. coli*. Isolate 81-1 was a strain of *Pantoea* spp. 2, which was isolated from a mixed culture with *E. coli*. It is very likely that these coliforms were only found on L-EMB agar due to their accidental transference with *E. coli* from EC broth with MUG.

#### 5.4.7. Confirmation of isolate identification

The MALDI-TOF MS confirmation of the API identifications was executed through a listing of the ten best matches when the ribosomal protein spectra of isolates were compared with the Bruker Taxonomy database. Only the three best matches and their corresponding identification values will be reported.

The possible advantage of MALDI-TOF MS over API is rooted in the subjectivity involved in interpreting the API test strip, whereas the MALDI Biotyper utilises spectra obtained from mass spectrometry and objectively compares it to spectra in the database. Furthermore, API 20E relies on identification of biochemical reactions. These reactions are phenotypic expressions of genetic characteristics, and may not be expressed in all strains of a species. It is also possible that environmental strains, such as these organisms which were isolated from river water, could have undergone adaptations which could impart phenotypic characteristics not conventionally associated with them. This would lead to weak or unacceptable identifications, since the API database has been compiled from the reactions of clinical specimens. The ribosomal proteins that are detected with the MALDI Biotyper, however, are highly conserved proteins (Kozak, 1983) which do not readily undergo changes.

#### 5.4.8. Confirmation of identification of ATCC reference strains of *E. coli*

The MALDI-TOF MS results for the four reference strains of *E. coli*, used as positive controls, are presented in Table 5.14. The various levels of identification associated with the different values reported can be seen in Table 5.3. Values between 2.300 and 3.000 denotes a high probability of species identification, while values falling between 2.000 and 2.299 denotes a secure identification of the genus and probable identification of the species.



**Table 5.10.** MALDI-TOF MS identification of four positive-control reference strains of *E. coli*

Strain	1 <sup>st</sup> best	Value	2 <sup>nd</sup> best	Value	3 <sup>rd</sup> best	Value
ATCC 11775	<i>E. coli</i>	2.499	<i>E. coli</i>	2.447	<i>E. coli</i>	2.422
ATCC 4350	<i>E. coli</i>	2.443	<i>E. coli</i>	2.365	<i>E. coli</i>	2.335
ATCC 10799	<i>E. coli</i>	2.440	<i>E. coli</i>	2.385	<i>E. coli</i>	2.347
ATCC 13135	<i>E. coli</i>	2.420	<i>E. coli</i>	2.416	<i>E. coli</i>	2.328

The results presented in Table 5.10 confirm the API identifications which indicated that these strains are cultures of *E. coli*. The identification values for the three best matches for all four organisms were above 2.300, which signifies highly probable species identification. Additionally, the confirmation of these organisms as *E. coli* is affirmation that some strains of *E. coli*, such as ATCC 4350 and ATCC 10799, cannot produce purple colonies on chromogenic agar (see Table 5.5).

#### 5.4.9. Confirmation of identification of isolates from LST broth

The MALDI-TOF MS results for isolates from LST broth are presented in Table 5.11, along with the corresponding identification values by the MALDI Biotyper.

**Table 5.11.** MALDI-TOF MS identification of organisms isolated from LST broth

Atypical reactions							
Isolate	1 <sup>st</sup> best	Value	2 <sup>nd</sup> best	Value	3 <sup>rd</sup> best	Value	API ID%
4	<i>A. hydrophila</i>	2.342	<i>A. hydro. spp. hydrophila</i>	2.341	<i>Aeromonas</i> spp.	2.295	85.9
5	<i>Entb. aerogenes</i>	2.377	<i>Entb. aerogenes</i>	2.297	<i>Entb. aerogenes</i>	2.211	99.0
11-1	<i>Citro. braakii</i>	2.407	<i>Citro. braakii</i>	2.388	<i>Citro. freundii</i>	2.259	67.8
11-2	<i>M. morgani</i>	2.565	<i>M. morg. spp. morgani</i>	2.532	<i>M. morg. spp. morgani</i>	2.503	99.9
12	<i>Citro. braakii</i>	2.257	<i>Citro. braakii</i>	2.213	<i>Citro. freundii</i>	2.208	
13-1	<i>E. coli</i>	2.355	<i>E. coli</i>	2.343	<i>E. coli</i>	2.335	99.9
13-2	<i>Prov. alcalifaciens</i>	2.409	<i>Prov. alcalifaciens</i>	2.393	<i>Prov. rettgeri</i>	1.786	73.8
14	<i>Prov. alcalifaciens</i>	2.488	<i>Prov. alcalifaciens</i>	2.481	<i>Prov. rustigianii</i>	2.025	71.7
15	<i>K. pneumoniae</i>	2.510	<i>K. pneum. spp. pneum.</i>	2.499	<i>K. pneumoniae</i>	2.455	63.6
17	<i>Entb. asburiae</i>	2.337	<i>Entb. kobei</i>	2.276	<i>Entb. spp.</i>	2.245	
21	<i>M. luteus</i>	2.260	<i>M. luteus</i>	2.119	<i>M. luteus</i>	2.064	99.5
26	<i>B. pseudofirmus</i>	1.740	<i>B. safensis</i>	1.676	<i>B. safensis</i>	1.633	
27	<i>K. pneum. spp. pneum.</i>	2.594	<i>K. pneumoniae</i>	2.478	<i>K. pneumoniae</i>	2.441	87.0
28	<i>K. pneum. spp. pneum.</i>	2.612	<i>K. pneumoniae</i>	2.522	<i>K. pneumoniae</i>	2.495	88.5

**Table 5.11 continued**

Table 5.11 continued

Atypical reactions							
Isolate	1 <sup>st</sup> best	Value	2 <sup>nd</sup> best	Value	3 <sup>rd</sup> best	Value	API ID%
30	<i>S. marcescens</i>	2.350	<i>S. marc. spp. marcescens</i>	2.283	<i>S. marcescens</i>	2.194	
35	<i>S. marcescens</i>	2.430	<i>S. marc. spp. marcescens</i>	2.261	<i>S. marcescens</i>	2.217	97.4
39	<i>H. alvei</i>	2.384	<i>H. alvei</i>	2.280	<i>H. alvei</i>	2.255	99.9
41	<i>Entb. cloacae</i>	2.339	<i>Entb. asburiae</i>	2.243	<i>Entb. cloacae</i>	2.227	95.0
42	<i>Entb. asburiae</i>	2.220	<i>Entb. asburiae</i>	2.213	<i>Entb. spp.</i>	2.202	
43	<i>Entb. asburiae</i>	2.237	<i>Entb. kobei</i>	2.139	<i>Entb. cloacae</i>	2.105	92.2
44	<i>Prov. rettgeri</i>	2.597	<i>Prov. rettgeri</i>	2.352	<i>Prov. rettgeri</i>	2.238	99.9
46	<i>K. pneumoniae</i>	2.447	<i>K. pneumoniae</i>	2.250	<i>K. pneum. spp. ozaenae</i>	2.246	83.5
47	<i>Citro. braakii</i>	2.343	<i>Citro. freundii</i>	2.339	<i>Citro. freundii</i>	2.268	99.8
48-1	<i>E. coli</i>	2.317	<i>E. coli</i>	2.315	<i>E. coli</i>	2.185	99.5
48-2	<i>S. marcescens</i>	2.329	<i>S. marc. spp. marcescens</i>	2.240	<i>S. marcescens</i>	2.180	69.6
50	<i>Entb. asburiae</i>	2.293	<i>Entb. kobei</i>	2.207	<i>Entb. spp.</i>	2.082	84.9
53	<i>K. pneumoniae</i>	2.352	<i>K. variicola</i>	2.231	<i>K. pneumoniae</i>	2.189	96.7
56	<i>Entb. cloacae</i>	2.253	<i>Entb. cloacae</i>	2.243	<i>Entb. cloacae</i>	2.090	95.0
59	<i>K. pneumoniae</i>	2.272	<i>K. pneumoniae</i>	2.259	<i>K. pneum. spp. pneum.</i>	2.239	73.2
80	<i>Citro. freundii</i>	2.411	<i>Citro. freundii</i>	2.395	<i>Citro. freundii</i>	2.360	96.7
86	<i>Raoul. ornithinolytica</i>	2.574	<i>Raoul. planticola</i>	2.263	<i>Raoul. ornithinolytica</i>	2.192	57.2
87	<i>Entb. kobei</i>	2.259	<i>Entb. asburiae</i>	2.200	<i>Entb. asburiae</i>	2.120	99.1
95	<i>E. coli</i>	2.406	<i>E. coli</i>	2.342	<i>E. coli</i>	2.289	64.4
Typical reactions							
Isolate	1 <sup>st</sup> best	Value	2 <sup>nd</sup> best	Value	3 <sup>rd</sup> best	Value	API ID%
101	<i>Prot. vulgaris</i>	2.492	<i>Prot. vulgaris</i>	2.490	<i>Prot. vulgaris</i>	2.480	99.9
109	<i>Prov. stuartii</i>	2.377	<i>Prov. stuartii</i>	2.346	<i>Prov. rettgeri</i>	1.481	53.4

The three best MALDI-TOF MS identifications for each isolate from LST broth given in Table 5.11 showed some variation for certain organisms in species identification, but all three results consistently agreed for the genus identification. The identification values for all isolates, except isolate 26, were very high, and exceeded 2.000 in at least the two best identifications for each organism.

#### Identification discrepancies

The identifications shown in Table 5.11 did not correspond with API identifications on genus level for 42.9% of the isolates. Of the isolates with identification discrepancies, all isolates with unacceptable API identification were identified with the MALDI Biotyper. They were found to be *Citrobacter braakii* (12), *Enterobacter asburiae* (17 and 42), *Bacillus pseudofirmus* (26) and *Serratia marcescens* (30). Consequently, isolates 12, 17, 30 and 42 belong to the coliform group

and should have produced gas in LST broth. This increases the number of coliforms isolated from LST broth to 29, and the number of coliform isolates causing under-estimation from 92.0% to 93.1%. Isolate 26, however, is a “typical organism” in LST according to the definition of the term for the study, since it is not a coliform and did not produce gas in the medium.

Six (11-1, 43, 53, 59, 86 and 95) isolates with discrepant identifications were identified as coliforms with API. Although the MALDI Biotyper assigned them to different genera, they were all grouped into genera within the coliform group once again. Therefore, their implications for LST broth, causing under-estimation, remain the same. A word should be said regarding isolate 86, which was identified as *Raoultella ornithinolytica*. This genus does not appear in the list of coliforms by Leclerc *et al.* (2001) and is not listed under *Enterobacteriaceae* in Bergey's Manual of Systematic Bacteriology (Brenner *et al.*, 2005). However, *Raoultella* was created due to the reclassification of species originally in the genus *Klebsiella* (i.e. *K. ornithinolytica*, *K. planticola* and *K. terrigena*) as proposed by Drancourt *et al.* (2001) after 16S rDNA and *rpoB* sequencing of *Klebsiella* species. Also, isolate 95, originally identified as belonging to *Enterobacter*, was identified by MALDI-TOF MS as *E. coli*. This explains the production of purple colonies on chromogenic agar by this isolate. The implications of anaerogenic *E. coli*, such as isolate 95, are far reaching: not only will it cause under-estimation of presumptive coliforms; but the organism would not be transferred to subsequent MTF steps to be enumerated as *E. coli*.

The remaining four isolates (21, 27, 46 and 50) with discrepant API identifications were originally identified as non-coliforms, but three (27, 46 and 50) were assigned to genera within the coliform group by the MALDI Biotyper. The resulted in an increase in the number of non gas-producing coliforms in LST broth, and will increase the number of coliform isolates causing under-estimation from 93.1% to 93.8%. The fourth isolate (21) was initially identified as *Chromobacterium violaceum*, and exhibited an unusual Gram reaction and morphology. The unexpected Gram positive reaction and coccus morphology which was recorded for this isolate agrees with the MALDI-TOF MS identification of the organism as *M. luteus*. The incorrect identification of this isolate with API is probably attributable to the inappropriate use of the API 20E test, which is specifically intended for the identification of enteric bacteria. For the method, however, the implication stays the same: *M. luteus* is not a coliform and did not produce gas in LST broth; therefore no anomalous reaction was caused.

#### Discriminatory ability of chromogenic agar for organisms isolated from LST broth

In the previous section dealing with chromogenic reactions of isolates from LST broth, some unusual reactions were observed. The production of pink colonies by non-coliform isolates 4, 46 and 50 were postulated to be due to enzymes capable of utilising dyes in the chromogenic mix. Since isolates 46 and 50 were identified by the MALDI Biotyper as coliforms, their discrepancies on chromogenic agar are negated. Isolate 4, however, was confirmed by MALDI-TOF MS to be *Aeromonas hydrophila*. Since this organism is not a coliform and produced pink colonies on the

medium, it must be concluded that chromogenic agar cannot differentiate some strains of *Aeromonas* from coliforms.

The eight isolates (11-2, 13-2, 14, 39, 44, 48-2, 101 and 109) identified as coliforms by API, which produced white colonies on chromogenic agar were initially suspected of being misidentified. Their API identifications were, however, all confirmed with the MALDI Biotyper, despite some isolates yielding low identification values (53.0% for isolate 109) with API 20E. Since misidentification was not the reason for the anomalous chromogenic reaction, it must be concluded that these organisms lack the enzymes required to utilise the dyes which cause a colour reaction. The isolates belong to the genera *Providencia*, *Proteus*, *Serratia*, *Hafnia* and *Morganella*. A similar phenomenon was noted in the section on chromogenic reactions of isolates obtained from the BGLB broth step. The genera implicated were *Proteus*, *Providencia*, *Morganella* and *Pantoea*. This is additional evidence supporting the theory that organisms from these genera cannot produce pink colonies on chromogenic agar. Furthermore, isolate 30, with an unacceptable API profile was identified by the Biotyper as belonging to the genus *Serratia*. This organism also produced white colonies on chromogenic agar.

Isolate 48-1 was identified by API as *E. coli* and produced pink colonies on chromogenic agar. The identification was confirmed by MALDI-TOF MS, and therefore it can be concluded that chromogenic agar cannot discriminate between coliforms and certain strains of *E. coli*. This is possibly due to the lack of functional  $\beta$ -D-glucuronidase within these strains.

Of the five API-unidentified organisms, four were identified by MALDI-TOF MS as coliforms. This explains the pink colonies produced by isolates 12, 17 and 42. Isolate 30, as mentioned previously, produced white colonies despite being of the genus *Serratia*, and this strain is presumably unable to cause a colour reaction on chromogenic agar. The fifth API-unidentified isolate (26) was identified (to probable genus level) by MALDI-TOF MS as *Bacillus pseudofirmus*. It has been demonstrated that some members of this genus possess  $\beta$ -D-galactosidase (Hirata *et al.*, 1985) and  $\beta$ -D-glucuronidase (Fricker *et al.*, 2010), and could consequently produce pink or purple colonies on chromogenic agar. However, the lack of colony production on chromogenic agar by this isolate indicates that the medium did not support the growth of this particular isolate. This could be attributable either to the absence of  $\beta$ -D-galactosidase and  $\beta$ -D-glucuronidase, or to incorrect identification with MALDI-TOF MS.

#### **5.4.10. Confirmation of identification of isolates from BGLB broth**

The MALDI-TOF MS results for isolates from BGLB broth and the corresponding identification values by the MALDI Biotyper are presented in Table 5.12.

**Table 5.12.** MALDI-TOF MS identification of organisms isolated from BGLB broth

Atypical reactions							
Isolate	1 <sup>st</sup> best	Value	2 <sup>nd</sup> best	Value	3 <sup>rd</sup> best	Value	API ID%
22	<i>M. morg. spp. morganii</i>	2.629	<i>M. morganii</i>	2.613	<i>M. morganii</i>	2.528	99.9
23	<i>M. morganii</i>	2.519	<i>M. morg. spp. morganii</i>	2.512	<i>M. morg. spp. morganii</i>	2.473	99.3
24-1	<i>Entb. asburiae</i>	2.359	<i>Entb. cloacae</i>	2.323	<i>Entb. kobei</i>	2.254	
24-2	<i>M. morg. spp. morganii</i>	2.655	<i>M. morganii</i>	2.647	<i>M. morg. spp. morganii</i>	2.572	99.9
25	<i>Entb. radicincitans</i>	2.053	<i>Entb. cowanii</i>	1.758	<i>E. coli</i>	1.740	95.0
29	<i>Raoul. ornithinolytica</i>	2.692	<i>Raoul. planticola</i>	2.331	<i>Raoul. planticola</i>	2.255	94.8
34	<i>Entb. asburiae</i>	2.221	<i>Entb. kobei</i>	2.159	<i>Entb. asburiae</i>	2.091	95.0
38	<i>K. oxytoca</i>	2.234	<i>Raoul. ornithinolytica</i>	2.125	<i>K. oxytoca</i>	2.024	97.9
40	<i>K. oxytoca</i>	2.320	<i>Raoul. ornithinolytica</i>	2.205	<i>K. oxytoca</i>	2.046	97.9
51	<i>Raoul. ornithinolytica</i>	2.653	<i>Raoul. planticola</i>	2.266	<i>Raoul. planticola</i>	2.211	94.8
75	<i>Raoul. ornithinolytica</i>	2.359	<i>Raoul. terrigena</i>	2.288	<i>Raoul. terrigena</i>	2.283	96.7
92	<i>Citro. braakii</i>	1.889	<i>Citro. koseri</i>	1.820	<i>Citro. koseri</i>	1.772	68.6
Typical reactions							
Isolate	1 <sup>st</sup> best	Value	2 <sup>nd</sup> best	Value	3 <sup>rd</sup> best	Value	API ID%
98	<i>Prov. stuartii</i>	2.277	<i>Prov. stuartii</i>	2.184	<i>Prot. mirabilis</i>	1.650	99.0
99	<i>Prot. vulgaris</i>	2.107	<i>Prot. vulgaris</i>	2.015	<i>Prot. vulgaris</i>	1.910	98.8
100	<i>Prot. mirabilis</i>	2.429	<i>Prot. mirabilis</i>	2.412	<i>Prot. mirabilis</i>	2.400	99.9
102	<i>Prot. vulgaris</i>	2.497	<i>Prot. penneri</i>	2.238	<i>Prot. vulgaris</i>	2.233	99.9
103	<i>Prot. mirabilis</i>	2.321	<i>Prot. mirabilis</i>	2.298	<i>Prot. mirabilis</i>	2.288	99.0
104	<i>Prov. stuartii</i>	2.333	<i>Prov. stuartii</i>	2.264	<i>Prov. alcalifaciens</i>	1.561	98.9
105	<i>Prot. mirabilis</i>	2.509	<i>Prot. mirabilis</i>	2.484	<i>Prot. mirabilis</i>	2.483	99.9
106	<i>Prot. mirabilis</i>	2.550	<i>Prot. mirabilis</i>	2.449	<i>Prot. mirabilis</i>	2.441	99.9
107	<i>Prot. vulgaris</i>	2.020	<i>Prot. vulgaris</i>	1.975	<i>Prot. vulgaris</i>	1.906	99.9
108	<i>K. pneum. spp. pneum.</i>	2.022	<i>K. pneum. spp. ozaenae</i>	1.879	<i>K. pneumoniae</i>	1.832	

The three best MALDI-TOF MS identifications for each isolate from BGLB broth showed some variation in species identification, but all three results agreed for the genus identification except in isolates 25, 38, 40 and 98. The identification values for all isolates were high and exceeded 2.000 in at least the two best identifications for each, except isolates 25 and 92.

### Identification discrepancies

The identifications shown in Table 5.12 disagreed with API identifications on genus level for 36.4% of the isolates. Of the isolates with identification discrepancies, both isolates with unacceptable API identification were identified with the MALDI Biotyper. They were found to belong to the genera *Enterobacter* (24-1) and *Klebsiella* (108). Since isolate 108 is a coliform and was isolated from a typical BGLB tube with gas production, this organism will have no negative impact on the method. Isolate 24-1, however, has been confirmed as a coliform, but was found in a BGLB tube with no gas production. Therefore, this organism will lead to an increase in the number of coliform isolates resulting in under-estimation from BGLB broth to 54.5%. Since it was co-isolated with *Morganella morganii* (24-2), it must be concluded that both strains are anaerogenic coliforms.

The six isolates with discrepant identifications (23, 29, 51, 75, 92 and 98) were all identified as coliforms with API. Although the MALDI Biotyper assigned them to different genera, they were all grouped in genera within the coliform group once again. Three of these isolates (29, 51 and 75) were identified as members of the genus *Raoultella*, which comprises strains originally belonging to *Klebsiella* (Drancourt *et al.*, 2001). Since these organisms were confirmed as coliforms according to the MALDI-TOF MS results, their implications for enumeration with BGLB broth remain the same. The five organisms (23, 29, 51, 75 and 92) isolated from atypical reactions will result in an under-estimation of the number of confirmed coliforms. Isolate 98 would be rightly be enumerated as a confirmed coliform.

### Discriminatory ability of chromogenic agar for organisms isolated from BGLB broth

When considering the API identifications of isolates from BGLB broth (Table 5.7), 65.0% of the identified coliforms produced white colonies on chromogenic agar. This number increased to 70.0% when the MALDI-TOF MS results were used, since isolate 108 was also confirmed to be a coliform. The coliform isolates producing white colonies on chromogenic agar were reported in section 5.4.4, with API identifications, to resort under the genera *Proteus*, *Providencia*, *Morganella* and *Pantoea*. With inclusion of the MALDI-TOF MS results, this list was amended to include *Citrobacter* (92) and exclude *Pantoea*. This list agrees well with the identified coliform genera from LST broth which produced white colonies on chromogenic agar.

#### 5.4.11. Confirmation of identification of isolates from EC broth with MUG

The MALDI-TOF MS results, with the corresponding identification values by the MALDI Biotyper, for isolates from EC broth with MUG are presented in Table 5.13. The results in the table show that the three best MALDI-TOF MS identifications for each isolate from EC broth with MUG showed some variation in species identification. However, all three results consistently agreed for the genus identification. The identification values for all isolates were high, and exceeded 2.000 in all three best match identifications for each organism, except isolate 64-2.

**Table 5.13.** MALDI-TOF MS identification of organisms isolated from EC broth with MUG

Atypical reactions							
Isolate	1 <sup>st</sup> best	Value	2 <sup>nd</sup> best	Value	3 <sup>rd</sup> best	Value	API ID%
6	<i>E. coli</i>	2.402	<i>E. coli</i>	2.373	<i>E. coli</i>	2.360	99.4
7	<i>E. coli</i>	2.350	<i>E. coli</i>	2.296	<i>E. coli</i>	2.274	97.7
8	<i>K. pneumoniae</i>	2.474	<i>K. pneum. spp. pneum.</i>	2.464	<i>K. pneumoniae</i>	2.414	99.4
9	<i>K. pneum. spp. pneum.</i>	2.118	<i>K. pneum. spp. pneum.</i>	2.109	<i>K. pneum. spp. ozaenae</i>	2.081	97.7
18	<i>E. coli</i>	2.404	<i>E. coli</i>	2.377	<i>E. coli</i>	2.334	99.5
19	<i>E. coli</i>	2.336	<i>E. coli</i>	2.245	<i>E. coli</i>	2.232	99.9
20	<i>K. pneum. spp. pneum.</i>	2.553	<i>K. pneumoniae</i>	2.493	<i>K. pneumoniae</i>	2.436	96.2
36	<i>Entb. asburiae</i>	2.269	<i>Entb. asburiae</i>	2.201	<i>Entb. kobei</i>	2.195	
37	<i>B. pseudofirmus</i>	1.673	<i>B. safensis</i>	1.629	<i>B. pseudofirmus</i>	1.617	
69	<i>Entb. asburiae</i>	2.256	<i>Entb. asburiae</i>	2.241	<i>Entb. spp.</i>	2.188	
70	<i>Entb. asburiae</i>	2.298	<i>Entb. asburiae</i>	2.253	<i>Entb. kobei</i>	2.194	
72	<i>Entb. kobei</i>	2.327	<i>Entb. asburiae</i>	2.244	<i>Entb. spp.</i>	2.186	
94	<i>Citro. freundii</i>	2.462	<i>Citro. freundii</i>	2.462	<i>Citro. freundii</i>	2.363	99.3
97	<i>Entb. asburiae</i>	2.255	<i>Entb. kobei</i>	2.217	<i>Entb. asburiae</i>	2.214	45.5
Typical reactions							
Isolate	1 <sup>st</sup> best	Value	2 <sup>nd</sup> best	Value	3 <sup>rd</sup> best	Value	API ID%
54	<i>E. coli</i>	2.348	<i>E. coli</i>	2.275	<i>E. coli</i>	2.269	99.8
55	<i>E. coli</i>	2.408	<i>E. coli</i>	2.367	<i>E. coli</i>	2.332	99.8
57-1	<i>E. coli</i>	2.361	<i>E. coli</i>	2.360	<i>E. coli</i>	2.298	99.5
57-2	<i>Entb. asburiae</i>	2.302	<i>Entb. asburiae</i>	2.243	<i>Entb. kobei</i>	2.147	59.2
60	<i>E. coli</i>	2.460	<i>E. coli</i>	2.413	<i>E. coli</i>	2.404	99.5
61	<i>E. coli</i>	2.476	<i>E. coli</i>	2.438	<i>E. coli</i>	2.416	98.3
62	<i>E. coli</i>	2.212	<i>E. coli</i>	2.135	<i>E. coli</i>	2.066	99.9
63	<i>E. coli</i>	2.364	<i>E. coli</i>	2.329	<i>E. coli</i>	2.285	50.0
64-1	<i>E. coli</i>	2.223	<i>E. coli</i>	2.182	<i>E. coli</i>	2.124	99.5
64-2	<i>Crono. sakazakii</i>	2.076	<i>Crono. sakazakii</i>	2.058	<i>Crono. sakazakii</i>	1.993	98.4
65	<i>E. coli</i>	2.323	<i>E. coli</i>	2.318	<i>E. coli</i>	2.316	99.5
66	<i>E. coli</i>	2.337	<i>E. coli</i>	2.334	<i>E. coli</i>	2.298	99.8
67	<i>E. coli</i>	2.286	<i>E. coli</i>	2.187	<i>E. coli</i>	2.184	99.8
68	<i>E. coli</i>	2.347	<i>E. coli</i>	2.322	<i>E. coli</i>	2.284	99.5
71	<i>Entb. asburiae</i>	2.229	<i>Entb. asburiae</i>	2.222	<i>Entb. asburiae</i>	2.217	63.8
73-1	<i>E. coli</i>	2.406	<i>E. coli</i>	2.315	<i>E. coli</i>	2.223	
73-2	<i>E. coli</i>	2.457	<i>E. coli</i>	2.396	<i>E. coli</i>	2.393	
73-3	<i>Prot. mirabilis</i>	2.354	<i>Prot. mirabilis</i>	2.349	<i>Prot. mirabilis</i>	2.317	

**Table 5.13 continued**



Table 5.13 continued

Typical reactions							
Isolate	1 <sup>st</sup> best	Value	2 <sup>nd</sup> best	Value	3 <sup>rd</sup> best	Value	API ID%
74	<i>E. coli</i>	2.357	<i>E. coli</i>	2.322	<i>E. coli</i>	2.313	99.5
79	<i>E. coli</i>	2.280	<i>E. coli</i>	2.274	<i>E. coli</i>	2.252	99.9
82	<i>E. coli</i>	2.384	<i>E. coli</i>	2.307	<i>E. coli</i>	2.299	99.8
84-1	<i>E. coli</i>	2.211	<i>E. coli</i>	2.205	<i>E. coli</i>	2.172	99.8
84-2	<i>E. coli</i>	2.365	<i>E. coli</i>	2.357	<i>E. coli</i>	2.353	99.9
85	<i>E. coli</i>	2.265	<i>E. coli</i>	2.223	<i>E. coli</i>	2.183	99.9
88	<i>Entb. asburiae</i>	2.311	<i>Entb. asburiae</i>	2.160	<i>Entb. asburiae</i>	2.155	
89	<i>S. marcescens</i>	2.438	<i>S. marc. spp. marcescens</i>	2.321	<i>S. marcescens</i>	2.317	99.8
90	<i>K. pneum. spp. pneum.</i>	2.568	<i>K. pneum. spp. pneum.</i>	2.547	<i>K. pneumoniae</i>	2.539	

#### Identification discrepancies in isolates from EC broth with MUG

The identifications shown in Table 5.13 disagreed with API identifications on genus level for 29.3% of the isolates. Of the isolates with identification discrepancies, all isolates with unacceptable API identification were identified with the MALDI Biotyper. Those isolated from atypical reactions were identified as belonging to *Enterobacter* (36, 69, 70 and 72) and *Bacillus* (37). Although the aforementioned four isolates identified as *Enterobacter* are coliforms, they are not thermotolerant coliforms. In the genus *Enterobacter*, only *Enterobacter aerogenes* and *Enterobacter cloacae* are considered to be thermotolerant coliforms (Leclerc *et al.*, 2001). It is, however, important to mention at this point that the MALDI Biotyper cannot yet effectively distinguish the species within the *Enterobacter cloacae* complex, which comprises *Enterobacter asburiae*, *Enterobacter cloacae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii* and *Enterobacter nimipressuralis* (Kostrzewa, 2010). Therefore, these organisms have no implications for the method, since they would not have been enumerated. Similarly, the presence of a member of the genus *Bacillus* is not troublesome for the enumeration of thermotolerant coliforms. There is, of course, a possibility that this organism could inhibit the growth of true test organisms (Evans *et al.*, 1981a; Edberg & Edberg, 1988), but there is no empirical evidence here to support this theory.

The organisms which were not identified with API and which were isolated from typical reactions were identified as belonging to *Enterobacter* (71 and 88), *Proteus* (73-3) and *Klebsiella* (90). *Klebsiella pneumoniae* is a thermotolerant coliform, and therefore its presence in a typical tube is not surprising. Its hydrolysis of MUG is a confounding factor, since this will result in a false-positive enumeration of *E. coli*, but should not be surprising since other workers have attributed this ability to *K. pneumoniae* as well (Chang *et al.*, 1989). The presence of isolates 71 and 88, *Enterobacter asburiae*, in typical tubes is, however, a problem. These organisms do not fall into the thermotolerant coliform group, and should not be able to grow at 44.5°C. If MUG was to be

omitted from the assay, this isolate would be incorrectly enumerated as a thermotolerant coliform *in situ*. Furthermore, its concurrent production of fluorescence from MUG, in this case, would have resulted in a false enumeration of *E. coli*. The implications of the presence of isolate 73-3 should be carefully considered. Since this organism was isolated from a tube where it was co-occurring with two strains of *E. coli* (73-1 and 73-2), it cannot be assumed that this organism was responsible for the typical reaction. It is most likely an unfortunate passenger, which was transferred from BGLB broth by accident. If the enumeration of this tube was done, it would have been correctly counted as *E. coli*, since *E. coli* was present, irrespective of the presence of *Proteus*.

There were two discrepant identifications (7 and 8) of organisms isolated from atypical reactions. Isolate 7 was initially identified as *K. pneumoniae*, but was shown by MALDI-TOF MS to be *E. coli*. In addition, isolate 8 was originally identified as *E. coli*, but was found to be *K. pneumoniae* when examined by MALDI-TOF MS. Since isolate 7 was isolated from a tube with growth, fluorescence and no gas production, this organism is presumably an anaerogenic strain of *E. coli*. Isolate 8 was isolated from a tube with growth, no fluorescence and no gas. Since *K. pneumoniae* is a thermotolerant coliform, it is very likely that this, too, is an anaerogenic strain. The implication of the presence of isolate 7 is an under-estimation of *E. coli* in EC broth with MUG. The presence of isolate 8 causes no detrimental effect in the assay as it was performed here, since thermotolerant coliforms were not enumerated and the failure by the organism to hydrolyse MUG excludes it as *E. coli*. If MUG is omitted from the assay, however, the organism would not be counted as a thermotolerant coliform, which would lead to under-estimation.

A single discrepant identification arose from organisms isolated from typical reactions. Isolate 61 was originally identified as *Kluyvera* spp., but was found to be *E. coli* by MALDI-TOF MS. This resulted in an increase in the proportion of true positive *E. coli* organisms, from 70.4% to 74.1% when MALDI-TOF MS results were used.

#### Discriminatory ability of chromogenic agar for organisms isolated from EC broth with MUG

It was observed in section 5.4.5, relating to chromogenic reactions of isolates from EC broth with MUG, that six isolates identified as *E. coli* produced pink colonies on chromogenic agar. With the inclusion of MALDI Biotyper results, this number is reduced to five, since isolate 8 was found to be *K. pneumoniae*, and not *E. coli*. This affirms the earlier theory that the production of pink colonies by a variety of *E. coli* strains cannot be coincidental, but instead, that some strains of *E. coli* cannot produce purple colonies on chromogenic agar. Therefore, chromogenic agar has limited discriminatory ability in distinguishing between some strains of *E. coli* and coliforms.

No identified non-*E. coli* coliform was able to produce purple colonies on chromogenic agar. One identified coliform (73-3) did, however, produce white colonies on chromogenic agar. It is, at this stage, no great surprise that this organism belongs to the genus *Proteus*, which has been observed in earlier sections to be unable to produce pink colonies on chromogenic agar.

#### 5.4.12. Confirmation of identification of isolates from L-EMB agar

The MALDI-TOF MS results for isolates from L-EMB agar are presented in Table 5.14, along with the corresponding identification values by the MALDI Biotyper.

**Table 5.14.** MALDI-TOF MS identification of organisms isolated from L-EMB agar

Atypical reactions							
Isolate	1 <sup>st</sup> best	Value	2 <sup>nd</sup> best	Value	3 <sup>rd</sup> best	Value	API ID%
1	<i>E. coli</i>	2.336	<i>E. coli</i>	2.256	<i>E. coli</i>	2.220	89.7
2	<i>K. pneumoniae</i>	2.420	<i>K. pneum. spp. pneum.</i>	2.378	<i>K. pneum. spp. ozaenae</i>	2.347	99.1
3-1	<i>E. coli</i>	2.454	<i>E. coli</i>	2.432	<i>E. coli</i>	2.416	97.7
3-2	<i>K. pneumoniae</i>	2.397	<i>K. pneumoniae</i>	2.317	<i>K. pneum. spp. pneum.</i>	2.289	99.8
16	<i>E. coli</i>	2.342	<i>E. coli</i>	2.319	<i>E. coli</i>	2.146	63.7
32	<i>Entb. asburiae</i>	2.344	<i>Entb. kobei</i>	2.253	<i>Entb. spp.</i>	2.186	99.1
45	<i>E. coli</i>	2.332	<i>E. coli</i>	2.291	<i>E. coli</i>	2.254	99.9
81-1	<i>E. coli</i>	2.477	<i>E. coli</i>	2.458	<i>E. coli</i>	2.385	
81-2	<i>K. pneumoniae</i>	2.465	<i>K. pneum. spp. pneum.</i>	2.345	<i>K. pneum. spp. ozaenae</i>	2.252	99.9
93	<i>Entb. kobei</i>	2.273	<i>Entb. asburiae</i>	2.247	<i>Entb. spp.</i>	2.180	99.9
Typical reactions							
Isolate	1 <sup>st</sup> best	Value	2 <sup>nd</sup> best	Value	3 <sup>rd</sup> best	Value	
31	<i>E. coli</i>	2.380	<i>E. coli</i>	2.235	<i>E. coli</i>	2.188	99.8
49	<i>K. pneum. spp. pneum.</i>	2.405	<i>K. pneum. spp. pneum.</i>	2.368	<i>K. pneumoniae</i>	2.287	
52	<i>E. coli</i>	2.127	<i>E. coli</i>	2.081	<i>E. coli</i>	2.001	99.9

The three best MALDI-TOF MS identification for each isolate from L-EMB agar showed some variation in species identification, but all three results consistently agreed for the genus identification. The identification values for all isolates were all high, and exceeded 2.000 in all three best match identifications for each organism.

#### Identification discrepancies

The identifications shown in Table 5.14 disagreed with API identification on genus level for 30.8% of the isolates. The typical reaction isolate (49) which could not be identified by API was identified convincingly (first best match = 2.405) by the MALDI Biotyper. This organism was identified as *K. pneumoniae*. Since it was isolated from an L-EMB plate with typical growth, the presence of this organism causes of two important implications: it would lead to an over-estimation of *E. coli* from EC broth with MUG; and it would be validated as *E. coli* on L-EMB agar. Since only positive tubes are streaked out on L-EMB, this organism must have grown and produced gas in EC broth and,

additionally, hydrolysed MUG. These reactions would lead to a false *E. coli* count and, consequently, over-estimation of *E. coli* numbers. Furthermore, since L-EMB agar is a validation step, the error in enumeration would not have been detected on L-EMB, due to the organism's growth as typical iridescent green colonies typical of *E. coli*.

An isolate (3-2) from an atypical reaction which could not be identified by API was identified by the MALDI Biotyper as *K. pneumoniae*. However, this organism's implications are not the same as those of isolate 49. Since it was isolated from a culture where it was mixed with *E. coli* (3-1), it was probably inadvertently transferred to L-EMB agar with *E. coli*. Similarly, the interpretation of its original EC broth with MUG tube as containing *E. coli* was entirely correct, since *E. coli* was present. However, it remains unclear, at this stage, whether this isolate assisted in the production of a typical reaction in EC broth with MUG.

The two other isolates which showed discrepant API and MALDI-TOF MS identifications (32 and 81-2) were both reclassified by the MALDI Biotyper results as belonging to coliform genera. Isolate 81-2 had been identified as a member of the coliform genus *Pantoea* with API, but was identified as *Klebsiella pneumoniae* based on its ribosomal protein spectrum. Isolate 32, however, was originally identified as *Pseudomonas aeruginosa*, but was identified by MALDI-TOF MS as being *K. pneumoniae*. The presence of these organisms is troubling, since it indicates that they were able to produce a typical reaction in EC broth with MUG to be enumerated as *E. coli*. The presence of *K. pneumoniae* is not surprising, however, since it does belong to the thermotolerant coliforms and it is known that some strains can produce fluorescence in the presence of MUG. *Enterobacter asburiae* is not designated as a thermotolerant coliform, and it is surprising to find it had grown and produced gas and fluorescence at 44.5°C in EC broth with MUG. It should be kept in mind, however, that the MALDI Biotyper cannot distinguish effectively between species within the *Enterobacter cloacae* complex (Kostrzewa, 2010). The implications arising from these two organisms are minimal for the method, however, if the atypical growth on L-EMB agar is used for the amendment of the *E. coli* enumeration done from EC broth with MUG.

#### Discriminatory ability of chromogenic agar for organisms isolated from L-EMB agar

The two organisms which were not identified by API both produced pink colonies on chromogenic agar. Since they were both identified as *K. pneumoniae*, their reaction on chromogenic agar is typical for that of coliforms. The only other anomalous reaction was the production of pink colonies by isolate 32, which was API identified as belonging to the genus *Pseudomonas*. Since this organism was identified by MALDI-TOF MS as being *Enterobacter asburiae*, a coliform, its reaction on chromogenic agar is also typical. Therefore, no anomalous reactions were observed on chromogenic agar for organisms isolated from L-EMB agar.

## 5.5. CONCLUSIONS

There are several implications for the MTF method arising from the results reported in this study. The primary implication is the under- and over-estimation caused by atypical organisms isolated from the four steps of the MTF method. Additionally, the limitations of the API 20E system in identifying environmental organisms, when compared with MALDI-TOF MS, were demonstrated. Finally, the lack of discriminatory ability of chromogenic agar in distinguishing between *E. coli*, coliforms and non-coliforms was shown.

### 5.5.1. Under- and over-estimation caused by isolates

The proportion of isolates causing false positive, false negative, true positive and true negative reactions for each step is shown in Table 5.15.

**Table 5.15** Proportion of isolates causing false positive, false negative, true positive and true negative reactions in the MTF method<sup>a</sup>

Step	Test for	Number of organisms <sup>a</sup>	Atypical <sup>b</sup>		Typical <sup>b</sup>	
			False positive	False negative	True positive	True negative
LST broth	Coliforms (presumptive)	35 (31.5)	0 (0.0)	30 (85.7)	2 (5.7)	3 (8.6)
BGLB broth	Coliforms (confirmatory)	22 (19.8)	0 (0.0)	12 (54.5)	10 (45.5)	0 (0.0)
EC broth with MUG	<i>E. coli</i>	41 (36.9)	8 (19.5)	4 (9.8)	19 (46.3)	10 (24.4)
L-EMB agar	<i>E. coli</i> (completion/validation)	13 (11.7)	1 (7.7)	5 (38.5)	2 (15.4)	5 (38.5)
<b>Total for column</b>		<b>111 (100.0)</b>	<b>8 (7.2)</b>	<b>51 (45.9)</b>	<b>34 (30.6)</b>	<b>18 (16.2)</b>

<sup>a</sup> numbers in brackets are percentages of the total number of organisms

<sup>b</sup> numbers in brackets are percentages of the number of organisms per broth/agar

It is clear from Table 5.15 that false negatives are a considerable problem in LST and BGLB broths, if the distribution of organisms in a test sample is similar to that of the isolates. Since the presumptive and, especially, confirmed coliform counts are used to monitor the efficacy of chlorine disinfection of water (Ashbolt *et al.*, 2001), such under-estimations could result in the release of water of poor microbiological quality into the environment. In addition, this under-estimation of coliforms could lead to ill-informed complacency when monitoring river water used in the irrigation of fresh produce.

The proportion of false positive and false negative isolates in EC broth, causing over- and under-estimation respectively, were much lower than those resulting in correct enumeration, i.e. true positives and true negatives. However, this step enumerates the most important faecal indicator, and the under-estimation (9.8%) of *E. coli* is particularly worrying since this could cause

the release of faecally contaminated water into the environment or produce results that prompt no corrective action where it should be taken. An example of this would be the regular monitoring of rivers used for irrigation, where state regulatory bodies may not take action based on inaccurate MTF results. Although over-estimation due to false positives is a concern from a methodological perspective, as well as potentially resulting in corrective action when no such actions are necessary, it is preferable to have a method which errs on the side of caution as opposed to one which under-estimates.

The under-estimation caused by false negative isolates from L-EMB agar is also troublesome, since the correct procedure when faced with atypical colonies on L-EMB agar is to remove the original EC broth with MUG tube from the *E. coli* enumeration. This would cause the same implications as under-estimation by EC broth with MUG. The over-estimation by false positives, although relatively small, can cause an increase in the number of *E. coli* initially enumerated using EC broth with MUG, since these tubes should be added and the *E. coli* enumeration amended. The implication of this is similar to those of over-estimation by EC broth with MUG, which results in the over-estimation of *E. coli* numbers.

It must be conceded here that the results in Table 5.15 are preliminary, as the MTF reactions assigned to isolates in this chapter are based on their tube of origin. In Chapter 8 the isolates discussed in this chapter will be evaluated individually to determine whether their true individual reactions correlate with the reactions they were isolated from. These results can be found in section 8.4.1 of this text.

The most common problematic genera causing false positives or false negatives for each step are listed in Table 5.16 in descending order of frequency. The number of incidences of error caused by a genus is indicated in brackets.

It is clear that the problematic genera shown in Table 5.16 are all members of the family *Enterobacteriaceae* and all belong to the coliform group. This is indicative of the efficacy of the method at excluding organisms which do not belong to the coliform group. Furthermore, the results in the table clearly illustrate the decrease in problematic genera for under-estimation as the MTF steps progress, which can be attributed to the increase in selective hurdles. In the last two steps, only *E. coli* can be under-estimated since the tests are specific for the enumeration of *E. coli*.

**Table 5.16** Problematic genera identified for each step of the MTF method

False negatives causing under-estimation			
LST broth	BGLB broth	EC broth with MUG	L-EMB agar
<i>Enterobacter</i> (8)	<i>Enterobacter</i> (3)	<i>Escherichia</i> (4)	<i>Escherichia</i> (5)
<i>Klebsiella</i> (6)	<i>Morganella</i> (3)		
<i>Citrobacter</i> (4)	<i>Raoultella</i> (3)		
<i>Escherichia</i> (3)	<i>Klebsiella</i> (2)		
<i>Providencia</i> (3)	<i>Citrobacter</i> (1)		
<i>Serratia</i> (3)			
<i>Hafnia</i> (1)			
<i>Morganella</i> (1)			
<i>Raoultella</i> (1)			
False positives causing over-estimation			
LST broth	BGLB broth	EC broth with MUG	L-EMB agar
		<i>Enterobacter</i> (3)	<i>Klebsiella</i> (1)
		<i>Cronobacter</i> (1)	
		<i>Klebsiella</i> (1)	
		<i>Proteus</i> (1)	
		<i>Serratia</i> (1)	

The results for false positives causing over-estimation indicate that no problematic genera for the over-estimation of coliforms were detected. However, five genera within the coliform group were implicated in the over-estimation of *E. coli*. Finally, three genera were identified as problematic both for over- as well as under-estimation. These genera are *Enterobacter*, *Klebsiella* and *Serratia*. If organisms belonging to these genera are in abundance in the test sample, it is to be expected that the degree of error for both under- and over-estimation by the method will be increased.

To date, the preference of *E. coli* over coliforms as the faecal indicator of choice in water microbiology (Edberg *et al.*, 2000) has been motivated primarily by its higher specific prevalence in faeces when compared to coliforms, which are ubiquitous in the environment (Wutor *et al.*, 2009). These results, however, provide additional methodological support for using *E. coli* instead of coliforms, since the latter group has been demonstrated here to be prone to considerable under-estimation with MTF. Despite this, the problematic genera for the enumeration of *E. coli* as shown in Table 5.16 confirm that the enumeration of this organism with MTF is also subject to error. More quantitative information relating to the microbiological composition of samples, and the prevalence of problematic genera, could provide greater insight into the magnitude of this problem when analysing a sample. Such insights could assist in more informed result assessments. It should be



noted however, that such information would remain site specific as microbiological population is not universal and would vary from site to site.

### 5.5.2. Efficacy of API and MALDI-TOF MS in identifying environmental isolates

The identification of the organisms isolated from the steps of the MTF media used to analyse river water with the API 20E system showed some inaccurate results. The biochemical identification was unable to identify 18 (16.2%) of the isolates. This may be due to the environmental strains undergoing some adaptations to better survive in their environment. Since the API database is mainly based on clinical strains, it is possible that the environmental organisms differed sufficiently to make identification impossible. Additionally, 21 (18.9%) of the isolates were misidentified with API and were reassigned to different genera by MALDI-TOF MS. These discrepant organisms were determined on genus level, and the percentage would increase if differences in species identification were included. The impact on the MTF method, however, can usually be determined from genus identification alone, except in the case of thermotolerant coliforms and differentiation of *E. coli* from other species within *Escherichia*.

The API 20E system has some limitations, especially when used for the identification of environmental isolates such as those in this study. This identification system is based on a limited set of characters (20 in the case of API 20E), which are based primarily on the biochemical reactions of clinical reference strains. Strain purity is also important to obtain correct, high-value identification with API 20E, but this requirement is equally important for high identification values with the MALDI Biotyper. MALDI-TOF MS-based methods also hold the added benefit of having the ability to identify bacteria from whole cells, crude cell fractions or suspensions (Lay Jr., 2001). In addition, the chemotaxonomic approach to identification can take as little as a few minutes, given that a considerable spectral library of bacterial strains is available (Lay Jr., 2001), and is able to distinguish between phylogenetically homologous organisms such as *E. coli* and *Shigella flexneri* (Lay Jr., 2001).

However, despite the benefits of MALDI-TOF MS and the limitations of API 20E, the latter is readily available and remains more practical and economically realistic to use in comparison with MALDI-TOF MS. The acquisition of a MALDI Biotyper instrument would cost approximately 1.5 million South African Rands, without considering running costs and updating of the Bruker Taxonomy library. In contrast, 100 API test strips and reagents costs approximately 8 000 South African Rands. Therefore, for the routine confirmation of isolates, API remains the more feasible alternative and produces acceptably reliable results.

### 5.5.3. Ability of chromogenic agar in distinguishing between *E. coli*, coliforms and non-coliforms

The *E. coli*/coliform chromogenic agar supplied by Oxoid, based on the detection of  $\beta$ -D-galactosidase and  $\beta$ -D-glucuronidase, which was used during this study demonstrated limited

discriminatory power with the isolates tested. The medium could not distinguish *E. coli* from coliforms in 9 of the 38 (23.7%) *E. coli* strains, including two positive-control ATCC reference strains. Furthermore, the agar could not distinguish coliforms from non-coliforms in 24 of 74 (32.4%) coliform isolates. The genera implicated here were *Citrobacter*, *Hafnia*, *Klebsiella*, *Morganella*, *Proteus* and *Providencia*. It is theorised that strains within these genera cannot utilise the dyes in the chromogenic mix due to a lack of functional  $\beta$ -D-galactosidase. Finally, one strain of *Bacillus pseudofirmus* was able to produce pink colonies on chromogenic agar, indicating the ability of some non-coliform organisms to utilise the dyes. The presence of  $\beta$ -D-galactosidase in *Bacillus stearothermophilus* has been reported (Hirata *et al.*, 1985), but these results indicate that *B. pseudofirmus* strains can also possess this enzyme and consequently elicit incorrect reactions on chromogenic agar. These organisms are thought to comprise a very small proportion of the non-coliforms, but more empirical evidence on the ability of non-coliforms to produce positive reactions on *E. coli*/coliform chromogenic agar is necessary to ascertain the true magnitude of the limitations caused by non-coliforms.

Aside from the limitations associated with *E. coli* and coliform identification, this medium is a very useful tool in determining the purity of *E. coli* and coliform isolates, since the purity of these organisms is very troublesome to establish due to difficulties in distinguishing between these organisms on a microscopic level. The visual judgment of colour between white, pink and purple colonies on this medium dramatically improved the ability to discern between pure and mixed *E. coli*/coliform colonies, and can lead to considerable savings in time and money by avoiding further analysis organisms which are still impure.

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## CHAPTER 6

### EVALUATION OF MONOPLEX AND MULTIPLEX PCR FOR DETECTING ENVIRONMENTAL COLIFORM AND *E. COLI* ISOLATES OBTAINED FROM MULTIPLE TUBE FERMENTATION OF RIVER WATER

#### 6.1. ABSTRACT

The accuracy of monoplex polymerase chain reaction (PCR) protocols for the detection of *Escherichia coli* and coliforms, as well as a multiplex PCR protocol for the detection of diarrheagenic *E. coli* pathotypes from a group (n=111) of environmental isolates obtained from river water multiple tube fermentation (MTF) analyses was evaluated here. The implications of the detection of *E. coli* from this group of isolates on the accuracy of the MTF method were also evaluated. The results of a monoplex PCR protocol evaluation revealed that the detection of coliforms using the *lacZ* protocol with primer annealing at 59.7°C gave unsatisfactory results with the group of isolates. A number of species falling within the coliform grouping, e.g. *Citrobacter braakii*, *Enterobacter asburiae*, *Serratia marcescens*, could not be reliably detected by this method, presumably due to the high primer annealing temperature. The evaluation of the *uidA* and *tuf* monoplex PCR protocols for the specific detection of *E. coli* showed that both protocols detected 100.0% of *E. coli* isolates. Additionally, the *uidA* protocol also correctly excluded all non-*E. coli* isolates. The *tuf* protocol, however, incorrectly detected two isolates belonging to *Enterobacter*, which can probably be attributed to a high homology between *E. coli* and *Enterobacter* for the *Tu* elongation factor. These results indicated that the *uidA* protocol would be the most specific and ubiquitous in the detection of *E. coli* from river water. Evaluation of the multiplex PCR protocol showed that the analysis was able to detect the genes of the five target pathotypes in reference strains. The protocol also identified one strain of enteroaggregative *E. coli* from the group of *E. coli* isolates (n=34). This low proportion was attributed to the loss of virulence factors after several sub-culturing steps, and the multiplex PCR protocol is considered to be appropriate for the detection of diarrheagenic pathotypes of *E. coli* from river water.

The results of the monoplex PCR analyses held limited implications for the MTF method, with only one strain of phenotypically identified *Klebsiella pneumoniae* being re-identified as *E. coli* through the PCR results. Consequently, this result increased the amount of false negative *E. coli* isolates. It was also determined that “false positive” *E. coli* isolates from *E. coli* (EC) broth with (4-methylumbelliferyl-β-D-glucuronide) MUG did not possess *uidA*. These isolates were presumably co-occurring with β-D-glucuronidase positive organisms which caused fluorescence with MUG, and it was concluded that these isolates could not result in false positive reactions in isolation.



## 6.2. INTRODUCTION

The determination of phenotypic characteristics of environmental isolates is important for identification and detection purposes. However, environmental isolates are notoriously difficult to identify to genus or species level using phenotypic characteristics (Boivin-Jahns *et al.*, 1995). In addition, the presence of genes in the genome of a bacterium does not necessarily guarantee their expression. Bej *et al.* (1991b) and Heijnen & Medema (2006) recommended that the detection of the *uidA* gene in *E. coli* is a more reliable identifier than the presence of  $\beta$ -D-glucuronidase, the enzyme for which it encodes. Boivin-Jahns *et al.* already in 1995 suggested that molecular identification of isolates reduces the incidence of misidentification when compared to classical phenotypic identification.

PCR has been shown to be an alternative for both the identification (Maheux *et al.*, 2009) and detection (Heijnen & Medema, 2006) of *E. coli* due to its sensitivity and specificity, while producing rapid results (Girones *et al.*, 2010). Additionally, the technique allows the detection of organisms in a viable but non-culturable state (Girones *et al.*, 2010). Since the PCR method is based on the amplification of a target gene or part of a gene, usually unique to the organism, the phenotypic expression or non-expression of genes is of no consequence for the efficacy of this technique. For this purpose, the detection of the *lacZ* gene has been proposed for the specific molecular identification of coliforms (Bej *et al.*, 1990) with results comparable to those generated by phenotypic methods (Bej *et al.*, 1991b). Genes for the specific detection of *E. coli* include the *uidA* gene (Heijnen & Medema, 2006), the *tuf* gene (Maheux *et al.*, 2009), associated with the Tu elongation factor (Schneider & Gibb, 1997), and the *mdh* gene associated with catalysing the action of malate dehydrogenase (Park *et al.*, 1995) in *E. coli*.

The detection of *E. coli* without further characterisation has also been criticised because of a lack of information about the degree of the risk posed by *E. coli* pathotypes through the implied faecal contamination (Ahmed *et al.*, 2007). It is well known that the *E. coli* species is not only a commensal indicator, but may also comprise pathogenic strains. The pathotypes are further subdivided into the enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and diffusely adherent *E. coli* (DAEC) (Moses *et al.*, 2006; Ahmed *et al.*, 2007; Carlos *et al.*, 2011). This collective is of particular importance from a public health point of view, since ingestion of these organisms could lead to serious, and sometimes fatal (Fagan *et al.*, 1999), enteric diseases.

Therefore, aside from the methodological significance of genes present in isolates from the MTF method, the pathogenicity of *E. coli* isolates is an important determinant of the risk posed by the river water from which these organisms were isolated. The infective dose for these diarrheagenic *E. coli* strains is considerably lower than that of their commensal counterparts



(Bhagwat, 2006) and, consequently, the health risk posed by irrigating MPFs with water containing pathotype strains of *E. coli* is dramatically amplified.

The aims of this study were: (1) to evaluate PCR protocols for distinguishing *E. coli* from coliforms and confirming phenotypic identification of these isolates; (2) to determine the implications of PCR identity confirmation on MTF accuracy and (3) to evaluate a PCR protocol for differentiating between diarrheagenic pathotype strains of *E. coli*.

### 6.3. MATERIALS AND METHODS

#### 6.3.1. Isolates and DNA isolation

A collection of 111 strains isolated from the four steps of the MTF method (Chapter 5) were used. Subsequent to phenotypic determination of their identities, the behaviour of the organisms in this method were categorised as “atypical” or “typical.”

The 111 organisms obtained from the four steps of the MTF method, four American Type Culture Collection (ATCC) *E. coli* reference strains (ATCC 11775, ATCC 4350, ATCC 10799 and ATCC 13135) and one reference strain each of *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae* and *Klebsiella pneumoniae* (used as positive controls), were streaked out on tryptose soy agar (TSA, Oxoid, Basingstoke, Hampshire, UK) and incubated overnight at 37°C. All organisms had previously been characterised, using the APIweb database, and identified by MALDI-TOF MS (see sections 5.4.3 through 5.4.12). DNA from the organisms was obtained by the boiling method described by Altalhi & Hassan (2009). A colony was suspended in 100 µL DNase- and RNase-free water in a sterile 1.5 mL centrifuge tube. The suspension was vortexed before placing in a waterbath at 100°C for 13 min to rupture cells. Tubes were placed on ice before centrifugation at 14 000 x *g* for 15 min in a Neofuge 13 centrifuge (Shanghai Lishen Scientific Equipment Company Limited, Shanghai, China). The resulting DNA-containing supernatant was transferred to a sterile Eppendorf tube and stored at -18°C. DNA samples were obtained in triplicate for all organisms.

#### 6.3.2. PCR evaluation of isolate identity

##### Monoplex PCR of isolates

Three PCR analyses were performed for the presence of the *tuf* and *uidA* genes (for *E. coli* detection) as well as the *lacZ* gene (for coliform detection). The *tuf* fragment (258 bp) was detected according to the primers and conditions used by Maheux and co-workers (2009). The presence of the *uidA* fragment (187 bp) was determined as described by Heijnen & Medema (2006). The primers were originally from Bej *et al.* (1991a), but were subsequently modified by Heijnen & Medema to decrease primer-dimer formation. The *lacZ* amplicon (264 bp) was detected

with primers described by Fricker & Fricker (1994). The primer sequences used in these PCR analyses are given in Table 6.1.

**Table 6.1** Size of monoplex PCR-assayed gene amplicons and sequences of primers used

Gene	Size (bp)	Sequence	Reference
<i>tuf</i>	258	F: 5'TGGGAAGCGAAAATCCTG3' R: 5'CAGTACAGGTAGACTTCTG3'	Maheux <i>et al.</i> (2009)
<i>uidA</i>	187	F: 5'ATGGAATTTGCGCGATTTTGC3' R: 5'ATTGTTTGCCTCCCTGCTGC3'	Heijnen & Medema (2006)
<i>lacZ</i>	264	F: 5'ATGAAAGCTGGCTACAGGAAGGCC3' R: 5'GGTTTATGCAGCAACGAGACCGTCA3'	Fricker & Fricker (1994)

Reaction tubes had a total reaction volume of 25  $\mu$ L, comprised of 0.5  $\mu$ L template DNA and PCR buffer (Kapa Biosystems, Cape Town, South Africa), magnesium chloride ( $MgCl_2$ ) (Kapa Biosystems, Cape Town, South Africa) and deoxyribonucleotide triphosphates (dNTPs) (Kapa Biosystems, Cape Town, South Africa) providing final concentrations of 0.025X, 2.5 mM and 0.2 mM, respectively. Forward and reverse primers for each reaction were added to obtain a final concentration of 0.4  $\mu$ M, and 0.625 U of hot start *Taq* polymerase (Kapa Biosystems, Cape Town, South Africa) was added per reaction volume.

Reaction tubes with template DNA from positive control reference strains, as well as a negative control tube with no DNA added, were included in every PCR analysis. Reaction tubes were placed into the G-Storm GS482 Thermal Cycler (G-Storm, Surrey, UK). The PCR programs for the analysis of the three genes are summarised in Table 6.2.

**Table 6.2** PCR programs used for the detection of the *tuf*, *uidA* and *lacZ* genes

Step	<i>tuf</i>	<i>uidA</i>	<i>lacZ</i>
Heated lid	110.0°C	110.0°C	110.0°C
Initialisation	95.0°C for 3 min	95.0°C for 3 min	95.0°C for 3 min
Denaturation <sup>‡</sup>	95.0°C for 30 sec	95.0°C for 30 sec	94.0°C for 30 sec
Annealing <sup>‡</sup>	58.0°C for 30 sec	60°C/59.7°C for 30 sec	59.7°C/60°C for 30 sec
Extension <sup>‡</sup>	72.0°C for 30 sec	72.0°C for 30 sec	72.0°C for 30 sec
Final elongation	72.0°C for 5 min	72.0°C for 5 min	72.0°C for 5 min
Storage	4.0°C for 5 min	4.0°C for 5 min	4.0°C for 5 min

<sup>‡</sup>35 cycles of denaturation, annealing and extension for all protocols

After PCR amplification, visualisation of products was done on agarose gels consisting of 1% w/v Lonza SeaKem® LE agarose (Whitehead Scientific, Cape Town, South Africa) in 0.5X Tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE) (Sigma-Aldrich, Kempton Park, South Africa) buffer. Ethidium bromide (Sigma-Aldrich, Kempton Park, South Africa) was incorporated

into the gels at a concentration of 0.01% (v/v). Electrophoresis was performed in a BG-Power 300 submarine system (Baygene Biotech Company Limited, Beijing, China), in the presence of 0.5X TBE buffer, at 210 V for 20 minutes.

Agarose gels were observed and recorded under UV light, with an InGenius gel documentation system (Syngene, Cambridge, UK) running the GeneSnap software (Syngene, Cambridge, UK). The appearance of a 187 bp, 258 bp or 264 bp band on the gel indicated the presence of the *uidA*, *tuf* or *lacZ* gene, respectively. The presence of the *lacZ* band was considered a molecular identification of the organism as a coliform. The presence of both *uidA* and *tuf* genes was considered a positive molecular identification of *E. coli*. Subsequent to any PCR analyses on isolated organisms, the *lacZ* protocol was tested on four reference coliform strains: *Citrobacter freundii*; *Enterobacter aerogenes*; *Enterobacter cloacae*; and *Klebsiella pneumoniae*. The *uidA* and *tuf* protocols were first tested on the four *E. coli* reference strains.

During the course of the discussion of these results, isolates will be designated as “true positive”, “true negative”, “false positive” and “false negative”. These designations inextricably link the identity of the isolate with its MTF reaction. The terms and their explanations are given in Table 6.3.

**Table 6.3** Isolate designations and their explanations

Designation	Coliforms	<i>E. coli</i>
“True positive”	A coliform isolate causing a positive reaction in the LST or BGLB step	An <i>E. coli</i> isolate causing a positive reaction in the EC with MUG or L-EMB step
“True negative”	A non-coliform isolate causing no reaction in the LST or BGLB step	A non- <i>E. coli</i> isolate causing no reaction in the EC with MUG or L-EMB step
“False positive”	A non-coliform isolate causing a positive reaction in the LST or BGLB step	A non- <i>E. coli</i> isolate causing a positive reaction in the EC with MUG or L-EMB step
“False negative”	A coliform isolate causing no reaction in the LST or BGLB step	An <i>E. coli</i> isolate causing no reaction in the EC with MUG or L-EMB step

#### *E. coli* pathotype multiplex PCR

The organisms identified with the *uidA* and *tuf* PCR as *E. coli* were subjected to the multiplex PCR to determine whether they are one of the five *E. coli* enteric pathogens (EAEC, EHEC, EIEC, EPEC or ETEC) associated with waterborne disease (Jagals *et al.*, 2006). The DNA of pure laboratory strains of the five pathotypes (kindly provided by Dr. T.G. Barnard, Water and Health Research Unit, University of Johannesburg) were obtained as described under section 6.3.1.

The PCR analysis was designed to detect the pathogenicity genes *eae*, *eagg*, *ial*, *lt*, *st*, *stx1*, *stx2* and the *mdh* housekeeping gene. The genes, their fragment sizes, associated *E. coli* pathotypes and primer sequences are shown in Table 6.4.

**Table 6.4** Size of multiplex PCR-assayed gene amplicons, their associated *E. coli* pathotypes and sequences of primers used in their detection

Gene	Size (bp)	<i>E. coli</i> pathotype	Sequence	Reference <sup>†</sup>
<i>stx2</i>	779	EHEC	F: 5'CCATGACAACGGACAGCAGTT3' R: 5'CCTGTCAACTGAGCACTTTG3'	Moses <i>et al.</i> (2006)
<i>ial</i>	630	EIEC	F: 5'GGTATGATGATGATG AGTCCA3' R: 5'GGAGGCCAACAATTATTTCC3'	López-Saucedo <i>et al.</i> (2003) Paton & Paton (1998)
<i>stx1</i>	614	EHEC	F: 5'ACACTGGATGATCTCAGTGG3' R: 5'CTGAATCCCCCTCCATTATG3'	Moses <i>et al.</i> (2006)
<i>lt</i>	450	ETEC	F: 5'GGCGACAGATTATACCGTG3' R: 5'CGGTCTCTATATTCCTGTT3'	Pass <i>et al.</i> (2000)
<i>eae</i>	384	EHEC EPEC	F: 5'GACCCGGCACAAGCATAAGC3' R: 5'CCACCTGCAGCAACAAGAGG3'	López-Saucedo <i>et al.</i> (2003)
<i>mdh</i>	300	All strains	F: 5'GGTATGGATCGTTCCGACCT3' R: 5'GGCAGAATGGTAACACCAGAGT3'	Tarr <i>et al.</i> (2002)
<i>eagg</i>	194	EAEC	F: 5'AGACTCTGGCGAAAGACTGTATC3' R: 5'ATGGCTGTCTGTAATAGATGAGAAC3'	Kong <i>et al.</i> (2002)
<i>st</i>	160	ETEC	F: 5' TTTCCCCTCTTTTAGTCAGTCAACTG3' R: 5'GGCAGGATTACAACAAAGTTCACA3'	Pass <i>et al.</i> (2000)

<sup>†</sup> Unless otherwise specified, references refer to both forward (F) and reverse (R) primer sequences

In the primer stock solution all primers, except for the *lt* forward and reverse primers, had a final concentration of 2 µM. The *lt* primers had a final concentration of 1 µM. A stock solution of standard culture mix (SCM) was prepared by combining 2 µL of DNA from EAEC, EHEC, EIEC, EPEC and ETEC laboratory strains.

The total reaction volume for the multiplex PCR analyses was 12.5 µL. This was comprised of 0.25 µL template DNA, Qiagen multiplex PCR mastermix (with hot start *Taq* polymerase) (Whitehead Scientific, Cape Town, South Africa) providing a final concentration of 3 mM MgCl<sub>2</sub>, and the primer stock solution providing a final concentration of 0.2 µM for all primers except *lt* (0.1 µM). A reaction tube containing 1.25 µL of the SCM mixture instead of normal template DNA was included in all multiplex PCR analyses. The SCM PCR product is essential, since it serves as a comparator during visualisation due to its presentation of all eight assayed genes.

Reaction tubes were placed into the G-Storm GS482 Thermal Cycler (G-Storm, Surrey, UK) and the multiplex PCR program was initialised. This program is summarised in Table 6.5, and was according to the conditions described by Omar & Barnard (2010).

**Table 6.5** Multiplex PCR program for the detection of five *E. coli* pathotypes

Step	Multiplex PCR
Heated lid	110 °C
Initialisation	95 °C for 15 min
Denaturation <sup>‡</sup>	94 °C for 30 sec
Annealing <sup>‡</sup>	57 °C for 90 sec
Extension <sup>‡</sup>	72 °C for 90 sec
Final elongation	72 °C for 10 min
Storage	4 °C for 5 min

<sup>‡</sup>35 cycles of denaturation, annealing and extension

PCR products were visualised on agarose gels consisting of 1.25% w/v Lonza SeaKem® LE agarose (Whitehead Scientific, Cape Town, South Africa) in 0.5X TBE buffer. Ethidium bromide (Sigma-Aldrich, Kempton Park, South Africa) was added to gels at a concentration of 0.01% (v/v). The gel was subjected to electrophoresis in a BG-Power 300 submarine system (Baygene Biotech Company Limited, Beijing, China), filled with 0.5X TBE buffer, at 120 V for 90 minutes.

Agarose gels were observed and recorded under UV light, with an InGenius gel documentation system (Syngene, Cambridge, UK) running the GeneSnap software (Syngene, Cambridge, UK). The detection of pathotypes was done on duplicate DNA samples from each organism. If a discrepancy arose, the third set of DNA was used for confirmation.

## 6.4. RESULTS AND DISCUSSION

### 6.4.1. PCR evaluation of isolate identity

Monoplex PCR: *lacZ*

Prior to performing *lacZ* PCR analyses on the isolates from the MTF steps, four coliform reference strains and four reference strains of *E. coli* were used to evaluate the value of the *lacZ* protocol in detecting coliforms. Two annealing temperatures were employed: results using 59.7 °C were unacceptable, and the *uidA* program with annealing at 60 °C was evaluated. The results of the *lacZ* PCR performed, at both temperatures, on these laboratory strains are shown in Table 6.6.

**Table 6.6** PCR results of four laboratory coliform strains analysed by *lacZ* PCR

Strain	Annealing step at 59.7°C	Annealing step at 60.0°C
<i>Citrobacter freundii</i>	(-)	(-)
<i>Enterobacter aerogenes</i>	-	-
<i>Enterobacter cloacae</i>	+	+
<i>Klebsiella pneumoniae</i>	(-)	(-)
<i>E. coli</i> (ATCC 11775)	+	+
<i>E. coli</i> (ATCC 4350)	(-)	Not tested
<i>E. coli</i> (ATCC 10799)	(-)	Not tested
<i>E. coli</i> (ATCC 13135)	+	+

(-) = unacceptably weak signal in the band region

It is clear from Table 6.6 that neither of the PCR programs produced reliable, convincing results. For annealing at 59.7°C, low-intensity bands were found in the amplification products of *C. freundii*, *K. pneumoniae*, ATCC 4350 and ATCC 10799. These bands were of such unacceptable intensity that they could not be used in practice to distinguish between coliforms and non-coliforms. No band was detected at the 264 bp *lacZ* position for *Enterobacter aerogenes*. These findings are in agreement with the results by Bej *et al.* (1990); who found that the *lacZ* PCR could not amplify the gene in *Enterobacter aerogenes* and *Citrobacter freundii*, and could not effectively detect *Klebsiella pneumoniae*, when the primer annealing temperature was raised to 50°C. Due to the non-detection and unacceptably weak band signals obtained for coliforms other than *E. coli* with both programs, two ATCC strains of *E. coli* were omitted from testing. This was done as the *lacZ* PCR had already proven to be ineffectual in the detection of coliforms, and the detection of *E. coli* was going to be performed using *E. coli*-specific PCR primers and protocols.

Some of the coliforms isolated from the MTF method were used to detect the presence of the *lacZ* gene (Table 6.7) using a PCR program with annealing at 59.7°C. As can be observed in Table 6.7, the *lacZ* PCR failed to detect 6 (42.9%) of the coliform isolates which were screened. However, the results are consistent with those in Table 6.6, which indicated that the PCR could detect *E. coli*. The PCR failed to detect isolates of *Citrobacter*, *Enterobacter* and *Serratia*. These results indicate that the list of organisms which cannot be detected by *lacZ* PCR with annealing at 50°C, according to Bej *et al.* (1990), can be extended to include *Citrobacter braakii*, *Enterobacter asburiae* and *Serratia marcescens*. The latter genus had already been identified in another study (Bej *et al.*, 1991b) as giving a negative reaction with *lacZ* if primer annealing occurred at 50°C. However, the results in Table 6.7 do contradict the finding by Bej *et al.* (1990) that *Klebsiella pneumoniae* cannot be detected by *lacZ* PCR. This could be attributed to the use of 50°C as annealing temperature by Bej *et al.* (1990), in comparison with the annealing temperature of 59.7°C used in the present study. Alternatively, the difference could ascribed to the characteristics

of the strains which were used, since the strain used in the study by Bej *et al.* (1990) was a ATCC reference strain and the strains tested here were environmental isolates. Based on these findings, it was decided that the *lacZ* PCR analysis was not reliable for the coliform identification.

**Table 6.7** Screening of coliform isolates using the *lacZ* PCR

Isolate	Identification	Presence of <i>lacZ</i>	Isolate	Identification	Presence of <i>lacZ</i>
7	<i>Escherichia coli</i>	+	71	<i>Enterobacter asburiae</i>	-
9	<i>K. pneum. spp. pneumoniae</i>	+	89	<i>Serratia marcescens</i>	-
20	<i>K. pneum. spp. pneumoniae</i>	+	12	<i>Citrobacter braakii</i>	-
54	<i>Escherichia coli</i>	+	17	<i>Enterobacter asburiae</i>	-
61	<i>Escherichia coli</i>	+	36	<i>Enterobacter asburiae</i>	-
63	<i>Escherichia coli</i>	+	42	<i>Enterobacter asburiae</i>	-
67	<i>Escherichia coli</i>	+	55	<i>Escherichia coli</i>	+

Four reference strains of *E. coli* and six *E. coli* isolates possessed the *lacZ* gene. These comprised five strains (54, 55, 61, 63 and 67) of *E. coli* which did produce gas in EC broth with MUG. However, a sixth strain (7) is an *E. coli* isolate which did not produce gas in EC broth with MUG, and is believed to be anaerogenic. This indicates that isolate 7 does possess the gene which encodes for the  $\beta$ -D-galactosidase enzyme (Ashbolt *et al.*, 2001), despite the exhibited anaerogenesis of the isolate. Therefore, if the production of gas from lactose is used as a criterion for phenotypic detection of *E. coli*, this organism would have been overlooked. Similarly, its inability to produce gas in EC broth with MUG would have resulted in its under-estimation in an *in situ* MTF.

#### Monoplex PCR: *uidA* and *tuf*

Two temperature protocols were evaluated using the four *E. coli* reference strains. The *uidA* PCR with annealing at 60°C gave unsatisfactory results, and a gradient was run to determine the optimal annealing temperature at 59.7°C. These results and preliminary screening of the *tuf* protocol are given in Table 6.8.

**Table 6.8** PCR results of *uidA* and *tuf* protocols using *E. coli* reference strains

Strain	<i>uidA</i> (Annealing at 60.0°C)	<i>uidA</i> (Annealing at 59.7°C)	<i>tuf</i>
ATCC 11775	(-)	+	+
ATCC 4350	(-)	+	+
ATCC 10799	(-)	+	+
ATCC 13135	(-)	+	+

(-) = unacceptably weak signal in the band region



Since the *uidA* PCR with annealing at 59.7°C and the *tuf* PCR were found to detect all *E. coli* reference strains and showed low levels of non-specific amplification, they were chosen as the most appropriate for the analysis of the isolates.

The results for “true positive” coliforms isolated from LST and BGLB broth steps are presented in Table 6.9.

**Table 6.9** PCR results for “true positive” isolates from LST and BGLB steps

Isolate	Identification	MTF step	<i>uidA</i>	<i>tuf</i>
101	<i>Proteus vulgaris</i>	LST	-	-
109	<i>Providencia stuartii</i>	LST	-	-
98	<i>Providencia stuartii</i>	BGLB	-	-
99	<i>Proteus vulgaris</i>	BGLB	-	-
100	<i>Proteus mirabilis</i>	BGLB	-	-
102	<i>Proteus vulgaris</i>	BGLB	-	-
103	<i>Proteus mirabilis</i>	BGLB	-	-
104	<i>Providencia stuartii</i>	BGLB	-	-
105	<i>Proteus mirabilis</i>	BGLB	-	-
106	<i>Proteus mirabilis</i>	BGLB	-	-
107	<i>Proteus vulgaris</i>	BGLB	-	-
108	<i>K. pneumoniae</i> spp. <i>pneumoniae</i>	BGLB	-	-

The results in Table 6.9 showed that these isolates did not possess the *uidA* or *tuf* genes. Since these isolates were not identified as *E. coli*, this was not surprising. The absence of the *uidA* gene from all the above isolates is encouraging for the accuracy of MTF enumeration of coliforms, since this gene encodes for  $\beta$ -D-glucuronidase. Therefore, these isolates, which would be transferred to the subsequent step based on a positive result, would not lead to false positive fluorescence reactions in the presence of the MUG included in the EC broth step.

No “false positive” coliform organisms were isolated from LST and BGLB steps. This phenomenon should not be interpreted as necessarily being indicative of the *in situ* absence of “false positive” coliforms in these two steps.

Results of the PCR analyses for “true negative” coliforms are given in Table 6.10. No “true negative” coliforms were isolated from the BGLB step. As with the “false positive” coliform results, this does not necessarily indicate that “true negative” organisms do not or would not occur in the BGLB step.

**Table 6.10** PCR results for “true negative” isolates from LST broth

Isolate	Identification	MTF step	<i>uidA</i>	<i>tuf</i>
4	<i>Aeromonas hydrophila</i>	LST	-	-
21	<i>Micrococcus luteus</i>	LST	-	-
26	<i>Bacillus pseudofirmus</i>	LST	-	-

The results in Table 6.10 show that none of the organisms possessed *uidA* or *tuf*. Since these organisms were not identified as *E. coli*, or even coliforms, the production of bands for *uidA* or *tuf* was not expected. The findings also confirm that these isolates cannot produce  $\beta$ -D-glucuronidase, and if they were inadvertently transferred with a true test organism to BGLB and then EC with MUG, they would not have been able to produce a false positive fluorescent reaction. Therefore, these isolates pose no threat to the accuracy of coliform and *E. coli* enumeration by MTF.

The results for “false negative” coliforms from LST and BGLB are shown in Table 6.11. This group is of particular interest as they would constitute an under-estimation of presumptive and confirmed coliforms in the MTF method. Figure 6.1 shows the *tuf* PCR analyses of several of these isolates as well as isolates of “true negative” coliforms (26), “true negative” *E. coli* (36) and “true positive” *E. coli* (73-1 and 73-2).

**Table 6.11** PCR results for “false negative” isolates from LST and BGLB steps

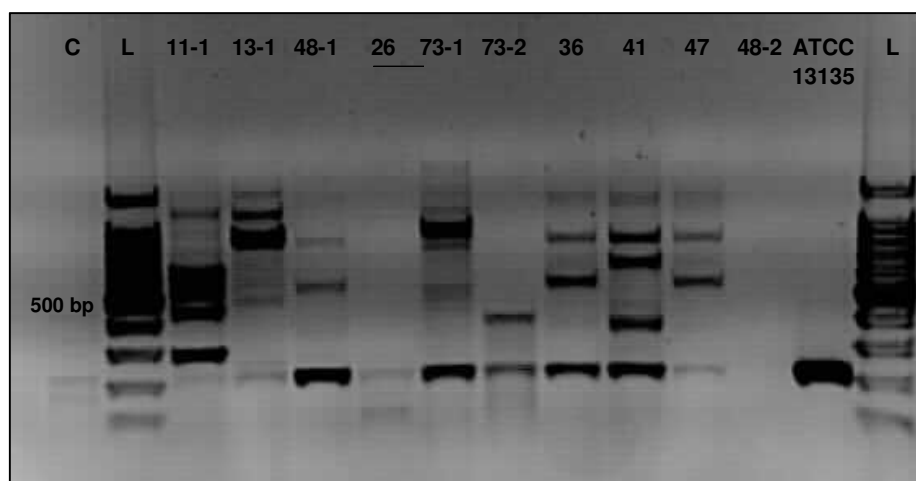
Isolate	Identification	MTF step	<i>uidA</i>	<i>tuf</i>
5	<i>Enterobacter aerogenes</i>	LST	-	-
11-1	<i>Citrobacter braakii</i>	LST	-	-
11-2	<i>Morganella morganii</i>	LST	-	-
12	<i>Citrobacter braakii</i>	LST	-	-
13-1	<i>Escherichia coli</i>	LST	+	+
13-2	<i>Providencia alcalifaciens</i>	LST	-	-
14	<i>Providencia alcalifaciens</i>	LST	-	-
15	<i>Klebsiella pneumoniae</i>	LST	-	-
17	<i>Enterobacter asburiae</i>	LST	-	-
27	<i>K. pneumoniae</i> spp. <i>pneumoniae</i>	LST	-	-
28	<i>K. pneumoniae</i> spp. <i>pneumoniae</i>	LST	-	-
30	<i>Serratia marcescens</i>	LST	-	-
35	<i>Serratia marcescens</i>	LST	-	-
39	<i>Hafnia alvei</i>	LST	-	-
41	<i>Enterobacter cloacae</i>	LST	-	+

**Table 6.11 continued**

**Table 6.11 continued**

Isolate	Identification	MTF step	<i>uidA</i>	<i>tuf</i>
42	<i>Enterobacter asburiae</i>	LST	-	-
43	<i>Enterobacter asburiae</i>	LST	-	-
44	<i>Providencia rettgeri</i>	LST	-	-
46	<i>Klebsiella pneumoniae</i>	LST	-	-
47	<i>Citrobacter freundii</i>	LST	-	-
48-1	<i>Escherichia coli</i>	LST	(+)	+
48-2	<i>Serratia marcescens</i>	LST	-	-
50	<i>Enterobacter asburiae</i>	LST	-	-
53	<i>Klebsiella pneumoniae</i>	LST	-	-
56	<i>Enterobacter cloacae</i>	LST	-	-
59	<i>Klebsiella pneumoniae</i>	LST	-	-
80	<i>Citrobacter freundii</i>	LST	-	-
86	<i>Raoultella ornithinolytica</i>	LST	-	-
87	<i>Enterobacter kobei</i>	LST	-	-
95	<i>Escherichia coli</i>	LST	+	+
22	<i>M. morganii</i> spp. <i>morganii</i>	BGLB	-	-
23	<i>Morganella morganii</i>	BGLB	-	-
24-1	<i>Enterobacter asburiae</i>	BGLB	-	-
24-2	<i>M. morganii</i> spp. <i>morganii</i>	BGLB	-	-
25	<i>Enterobacter radicincitans</i>	BGLB	-	-
29	<i>Raoultella ornithinolytica</i>	BGLB	-	-
34	<i>Enterobacter asburiae</i>	BGLB	-	-
38	<i>Klebsiella oxytoca</i>	BGLB	-	-
40	<i>Klebsiella oxytoca</i>	BGLB	-	-
51	<i>Raoultella ornithinolytica</i>	BGLB	-	-
75	<i>Raoultella ornithinolytica</i>	BGLB	-	-
92	<i>Citrobacter braakii</i>	BGLB	-	-

(+) = weak positive reaction



**Figure 6.1** *tuf* gel with PCR products of several “false negative” coliform isolates (11-1, 13-1, 41, 47, 48-1 and 48-2) as well as “true negative” coliform (26), “true negative” *E. coli* (36) and “true positive” *E. coli* (73-1 and 73-2) isolates. Lanes “C” and “ATCC 13135” show negative and positive controls, respectively, and lanes “L” show 100 bp ladders.

Ninety-seven percent (41 out of 42) of isolates in Table 6.11 gave expected results, with non-*E. coli* coliforms showing no bands and three *E. coli* strains showing both *uidA* and *tuf* bands. Isolate 41, *Enterobacter cloacae*, exhibited an interesting positive result with the *tuf* PCR despite a convincing identification using both API 20E and MALDI-TOF MS methods. This could possibly be attributed to the co-occurrence of a  $\beta$ -D-glucuronidase negative *E. coli* strain with this isolate, since a large number of *E. coli* isolates grew as pink colonies on chromogenic agar (see sections 5.3.2 and 5.3.4). Alternatively, it could also be due to a limitation of the primer set, however, no such limitations have been reported with *Enterobacter cloacae*. Maheux *et al.* (2008) did report a lack of specificity of the primers directed towards *Escherichia fergusonii* and *Shigella*, but both these species are genetically very similar (Brenner *et al.*, 2005; Maheux *et al.*, 2009) to *E. coli*.

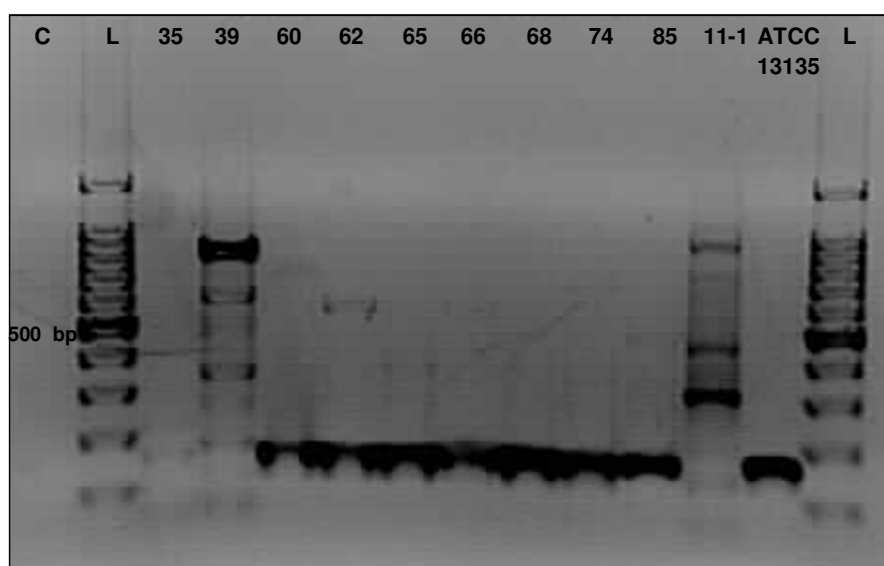
It should be reiterated that the large number of coliforms isolated (Chapter 5) from atypical LST and BGLB reactions should be a cause for concern. Even though it is expected that their proportion *in situ* is considerably less than that of this study, and that large numbers were isolated due to bias towards isolating from atypical reactions, they remain a risk for under-estimation of coliforms. Also, three isolates, which constituted 7.1% of false negative coliforms from the LST and BGLB steps and were not subjected to any bias within this group, are *E. coli* strains. In addition, if isolate 41 is a strain of *E. coli*, as indicated by the *tuf* PCR (see Figure 6.1), the number of *E. coli* isolates in this group would increase to four or 9.5% of false negative coliform isolates. The presence of this species would not only result in under-estimation of coliforms, but would also cause under-estimation of *E. coli*, since they would not be transferred to subsequent steps. The

*uidA* and *tuf* PCR results serve to confirm the identification of isolates 13-1, 48-1 and 95 as *E. coli*, and are additional proof of the under-estimation of *E. coli* by MTF. In addition, these results show that this under-estimation does not only occur in the later steps of the MTF (EC and L-EMB), but can be problematic in the earlier steps of the method. However, if these organisms were detected in LST and BLGB, the presence of *uidA* in their genome indicate that they would presumably possess the ability to produce  $\beta$ -D-glucuronidase and be detected in EC broth with MUG.

The results for “true positive” *E. coli* isolated from the EC with MUG and L-EMB steps are presented in Table 6.12. Figure 6.2 shows the *uidA* PCR gel for *E. coli* ATCC 13135, “false negative” coliform isolates 11-1, 35, 39 and the “true positive” *E. coli* isolates 60, 62, 65, 66, 68, 74 and 85.

**Table 6.12** PCR results for “true positive” isolates from the EC with MUG and L-EMB steps

Isolate	Identification	MTF step	<i>uidA</i>	<i>tuf</i>
54	<i>Escherichia coli</i>	EC + MUG	+	+
55	<i>Escherichia coli</i>	EC + MUG	+	+
57-1	<i>Escherichia coli</i>	EC + MUG	+	+
60	<i>Escherichia coli</i>	EC + MUG	+	+
61	<i>Escherichia coli</i>	EC + MUG	+	+
62	<i>Escherichia coli</i>	EC + MUG	+	+
63	<i>Escherichia coli</i>	EC + MUG	+	+
64-1	<i>Escherichia coli</i>	EC + MUG	+	+
65	<i>Escherichia coli</i>	EC + MUG	+	+
66	<i>Escherichia coli</i>	EC + MUG	+	+
67	<i>Escherichia coli</i>	EC + MUG	+	+
68	<i>Escherichia coli</i>	EC + MUG	+	+
73-1	<i>Escherichia coli</i>	EC + MUG	+	+
73-2	<i>Escherichia coli</i>	EC + MUG	+	+
74	<i>Escherichia coli</i>	EC + MUG	+	+
79	<i>Escherichia coli</i>	EC + MUG	+	+
82	<i>Escherichia coli</i>	EC + MUG	+	+
84-1	<i>Escherichia coli</i>	EC + MUG	+	+
84-2	<i>Escherichia coli</i>	EC + MUG	+	+
85	<i>Escherichia coli</i>	EC + MUG	+	+
31	<i>Escherichia coli</i>	L-EMB	+	+
52	<i>Escherichia coli</i>	L-EMB	+	+



**Figure 6.2** *uidA* gel with PCR products of “false negative” coliform isolates (35, 39 and 11-1) and “true positive” *E. coli* isolates (60, 62, 65, 66, 68, 74 and 85). Lanes “C” and “ATCC 13135” show negative and positive controls, respectively, and lanes “L” show 100 bp ladders.

All the isolates in Table 6.12 were identified as *E. coli* using the MALDI-TOF MS method, with PCR results confirming these findings. The presence of *uidA* in these isolates indicates that they should have the ability to produce  $\beta$ -D-glucuronidase (Feng *et al.*, 1991); as was the case for the “true positive” *E. coli* isolates from EC with MUG, since all these isolates grew, produced gas and produced fluorescence from MUG. However, this is not the case for all *E. coli* strains in nature and a review by Rompré and co-workers (2002) reported that the lack of  $\beta$ -D-glucuronidase in at least *E. coli* O157:H7. In addition, they also reported that *uidA* is present in some strains of *E. coli*, but remains unexpressed (Rompré *et al.*, 2002). They concluded that in some *E. coli* strains which do not fluoresce, the gene is not necessarily absent but is simply not expressed.

This conclusion cannot be drawn from the results of two isolates (31 and 52) from L-EMB, since their reaction in the EC with MUG step was not investigated. This is reported on in a later stage of the text (see section 8.4.1), where it was confirmed that both these isolates possess the ability to hydrolyse MUG. Since they were isolated from the validation step after EC broth with MUG, however, it is reasonable to believe that they had given a typical reaction in this medium and did produce fluorescence. This would agree with the presence of the *uidA* gene in both these organisms. The results presented in Table 6.12 confirm, at a molecular level, that all these isolates listed are “true positive” *E. coli* isolates.

The results for “false positive” *E. coli* isolates from the EC with MUG and L-EMB steps are given in Table 6.13.

**Table 6.13** PCR results for “false positive” isolates from EC with MUG and L-EMB steps

Isolate	Identification	MTF step	<i>uidA</i>	<i>tuf</i>
57-2	<i>Enterobacter asburiae</i>	EC + MUG	-	-
64-2	<i>Cronobacter sakazakii</i>	EC + MUG	-	-
71	<i>Enterobacter asburiae</i>	EC + MUG	-	-
73-3	<i>Proteus mirabilis</i>	EC + MUG	-	-
88	<i>Enterobacter asburiae</i>	EC + MUG	-	-
89	<i>Serratia marcescens</i>	EC + MUG	-	-
90	<i>K. pneum. spp. pneumoniae</i>	EC + MUG	-	-
49	<i>K. pneum. spp. pneumoniae</i>	L-EMB	-	-

The isolates in Table 6.13 are of particular interest, since they can be responsible for the over-estimation of *E. coli* with the MTF method. None were identified as *E. coli* or possessed the *uidA* or *tuf* genes, although they gave typical reactions during EC with MUG and L-EMB steps.

The isolate (49) from the L-EMB step was also negative for *uidA* or *tuf*. Since it was isolated from L-EMB agar, it must have been transferred from a typical EC with MUG tube which exhibited fluorescence. This is further evidence of the phenomenon of inadvertent transference of the anaerogenic organisms when co-occurrence with gas-producing organisms takes place.

The PCR results in Table 6.13 confirm the designation of the isolates listed as “false positive” for *E. coli* in the MTF method, as their identities were confirmed as non-*E. coli* by a molecular technique. These types of isolates appear to be responsible for over-estimation of *E. coli* by the MTF method, and can presumably cause these false positive reactions without the *uidA* or *tuf* genes in their genome. This is an interesting observation, since the *uidA* gene is responsible for  $\beta$ -D-glucuronidase production, but was not present in these isolates. Therefore, the fluorogenic capability of these isolates may be due to co-occurring organisms in the MTF reaction or, more unlikely, the possession of a different enzyme capable of hydrolysing MUG. The results for “true negative” *E. coli* isolates are given in Table 6.14.

**Table 6.14** PCR results for “true negative” *E. coli* isolates from EC with MUG and L-EMB steps

Isolate	Identification	MTF step	<i>uidA</i>	<i>tuf</i>
8	<i>Klebsiella pneumoniae</i>	EC + MUG	-	-
9	<i>K. pneum. spp. pneumoniae</i>	EC + MUG	-	-
20	<i>K. pneum. spp. pneumoniae</i>	EC + MUG	+	+
36	<i>Enterobacter asburiae</i>	EC + MUG	-	+
37	<i>Bacillus pseudofirmus</i>	EC + MUG	-	-

**Table 6.14 continued**



**Table 6.14 continued**

Isolate	Identification	MTF step	<i>uidA</i>	<i>tuf</i>
69	<i>Enterobacter asburiae</i>	EC + MUG	-	-
70	<i>Enterobacter asburiae</i>	EC + MUG	-	-
72	<i>Enterobacter kobei</i>	EC + MUG	-	-
94	<i>Citrobacter freundii</i>	EC + MUG	-	-
97	<i>Enterobacter asburiae</i>	EC + MUG	-	-
2	<i>Klebsiella pneumoniae</i>	L-EMB	-	-
3-2	<i>Klebsiella pneumoniae</i>	L-EMB	-	-
32	<i>Enterobacter asburiae</i>	L-EMB	-	-
81-2	<i>Klebsiella pneumoniae</i>	L-EMB	-	-
93	<i>Enterobacter kobei</i>	L-EMB	-	-

These “true negative” results were expected for isolates which were not *E. coli*. Two exceptions were found; the first was the presence of both *uidA* and *tuf* in isolate 20, which had been identified as *K. pneumoniae* spp. *pneumoniae*. There are two major problems with this identification as this isolate was from an EC with MUG tube which exhibited no fluorescence. This indicates that the *uidA* present in the organism is either unexpressed (Rompré *et al.*, 2002) or encodes for non-functional  $\beta$ -D-glucuronidase (Feng *et al.*, 1991). Secondly, the presence of *uidA* in this *Klebsiella* isolate is different to the *K. pneumoniae* spp. *pneumoniae* isolate strain (90) in Table 6.13 which caused false positive reactions in the EC step and did not possess the *uidA* gene, indicating that isolate 20 may not belong to *Klebsiella*. This is in agreement with reported results which showed that *Klebsiella* possesses no close homolog to the *E. coli uidRABC*, the operon where *uidA* is located (Blattner *et al.*, 1997; McClelland *et al.*, 2000) and that *Klebsiella pneumoniae* showed only 55.0% gene homology with *E. coli* K-12 (McClelland *et al.*, 2000).

The second exception was isolate 36, *Enterobacter asburiae* which showed a *tuf* band (see Figure 6.1), but was negative for *uidA*. Since both isolate 20 and 36 had undergone purification on nutrient agar; were examined biochemically and microscopically for purity; and grew as pure pink colonies on chromogenic agar, they were considered pure. However, several strains of *E. coli* were found to produce pink colonies on chromogenic agar (Chapter 5), and similar strains may have unobtrusively occurred with these isolates. Alternatively, these results could possibly allude to limitations in the specificity of the *tuf* PCR analysis.

The results of the remaining 13 isolates (Table 6.14) confirm that their identifications as organisms other than *E. coli* were correct. It must be noted that five isolates (2, 3-2, 32, 81-2 and 93) from the L-EMB step did not possess *uidA*. This is of interest as they had to produce positive reactions, including fluorescence, in EC with MUG to have been transferred to the L-EMB step. It should also be noted that isolates 3-2 and 81-2 were most probably inadvertently enumerated and transferred along with their co-habiting *E. coli* strain. This may also have been the case with the other three strains.

These results, apart from the discrepancies of isolates 20 and 36, confirm the designation of this group of isolates as “true negative” for *E. coli* in the MTF method. Such isolates would not have an adverse effect on the accuracy of the method.

The results of the “false negative” *E. coli* isolates from the EC broth and L-EMB steps are given in Table 6.15. This group is important for the accuracy of the MTF, as these organisms can lead to the under-estimation of *E. coli* numbers.

**Table 6.15** PCR results for “false negative” isolates from EC with MUG and L-EMB steps

Isolate	Identification	MTF step	<i>uidA</i>	<i>tuf</i>
6	<i>Escherichia coli</i>	EC + MUG	+	+
7	<i>Escherichia coli</i>	EC + MUG	+	+
18	<i>Escherichia coli</i>	EC + MUG	+	+
19	<i>Escherichia coli</i>	EC + MUG	+	+
1	<i>Escherichia coli</i>	L-EMB	+	+
3-1	<i>Escherichia coli</i>	L-EMB	+	+
16	<i>Escherichia coli</i>	L-EMB	+	+
45	<i>Escherichia coli</i>	L-EMB	+	+
81-1	<i>Escherichia coli</i>	L-EMB	+	+

The results in Table 6.15 were anticipated, since these isolates were all identified as *E. coli*, and it was expected that both *uidA* and *tuf* would be present. What was surprising was the presence of *uidA* in isolates 18 and 19, both which did not fluoresce in the EC with MUG step. Therefore, it is concluded that they could not express *uidA*, or possibly produce non-functional  $\beta$ -D-glucuronidase (Feng *et al.*, 1991).

Since the isolates from the L-EMB step had to have given a typical reaction, including fluorescence in the EC step, it is probable that *uidA* was present and also expressed phenotypically during the latter step of the MTF. These results confirm the designation of the organisms in Table 6.14 as “false negative” *E. coli* isolates, which will contribute to the under-estimation of *E. coli* during MTF enumeration.

#### 6.4.2. Impact of molecular identification on distribution of atypical and typical organisms

The only discrepancies in identification confirmation with phenotypic results were the presence of the *tuf* gene in two isolates (36, *Enterobacter asburiae* and 41, *Enterobacter cloacae*). The presence of *uidA*, *tuf* and *mdh* in isolate 20 (*Klebsiella pneumoniae* spp. *pneumoniae*) and the absence of *mdh* in isolate 6, which was identified as *E. coli*. Subsequent to multiplex PCR analysis, isolates 36 and 41 remained as strains of *Enterobacter*, the genus to which they were originally identified using phenotypic characteristics. Isolate 20 was re-identified as a strain of *E. coli* with unique biochemical characteristics when the molecular, phenotypic and ribosomal protein

data provided by PCR analyses, API 20E and MALDI-TOF MS, respectively, were considered. Therefore, the only change made to the categorisation of isolates as *E. coli* and non-*E. coli* was the inclusion of isolate 20 in the former category.

The proportion of atypical and typical isolates after this reclassification is depicted in Table 6.16. Numbers and proportional percentages shown in bold were affected and differ from those given in Table 5.15 (Chapter 5).

**Table 6.16.** Proportion of atypical and typical organisms after reclassification based on molecular identification

Step	Test for	Number of organisms <sup>a</sup>	Atypical <sup>b</sup>		Typical <sup>b</sup>	
			False positive	False negative	True positive	True negative
LST broth	Coliforms (presumptive)	35 (31.5)	0 (0.0)	30 (85.7)	2 (5.7)	3 (8.6)
BGLB broth	Coliforms (confirmatory)	22 (19.8)	0 (0.0)	12 (54.5)	10 (45.5)	0 (0.0)
EC broth with MUG	<i>E. coli</i>	41 (36.9)	8 (19.5)	<b>5 (12.2)↑</b>	19 (46.3)	<b>9 (21.9)↓</b>
L-EMB agar	<i>E. coli</i> (completion/validation)	13 (11.7)	1 (7.7)	5 (38.5)	2 (15.4)	5 (38.5)
<b>Total for column</b>		<b>111 (100.0)</b>	<b>8</b>	<b>51</b>	<b>34</b>	<b>18</b>

<sup>a</sup> numbers in brackets are percentages of total organisms

<sup>b</sup> numbers in brackets are percentages of total organisms per row

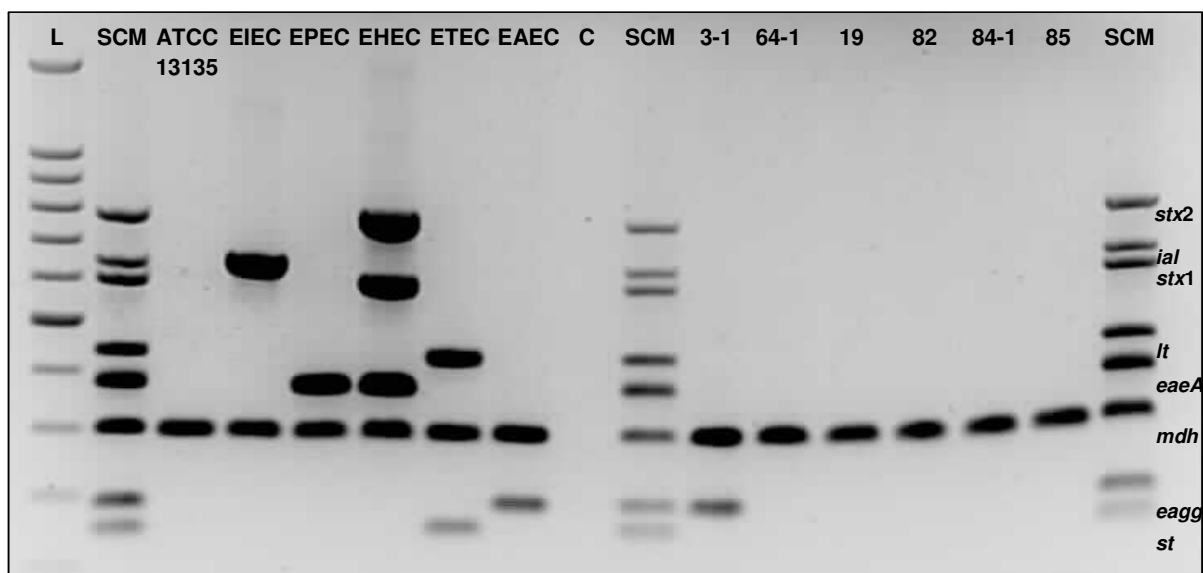
#### 6.4.3. *E. coli* diarrheagenic pathotype determination

##### Multiplex PCR

Thirty-four of the 111 isolates from the MTF steps were phenotypically identified as *E. coli* and this was confirmed by monoplex PCR through the presence of *uidA* and *tuf*. In addition, three other isolates (20, 36 and 41) exhibited either one or both of these genes. These 37 isolates were then subjected (Table 6.17 and Figure 6.3) to the multiplex PCR to determine whether they are *E. coli* diarrheagenic pathotypes. The presence of the *mdh* housekeeping gene was used as an additional confirmation of the identification as *E. coli*, but it is not an indication of pathogenicity.

**Table 6.17** Results of *E. coli* intestinal pathotype multiplex PCR done on 37 isolates

Isolate	Gene(s) detected	Isolate	Gene(s) detected
1	<i>mdh</i>	64-1	<i>mdh</i>
3-1	<i>eagg</i> , <i>mdh</i>	65	<i>mdh</i>
6	<i>mdh</i>	66	<i>mdh</i>
7	<i>mdh</i>	67	<i>mdh</i>
13-1	<i>mdh</i>	68	<i>mdh</i>
16	<i>mdh</i>	73-1	<i>mdh</i>
18	<i>mdh</i>	73-2	<i>mdh</i>
19	<i>mdh</i>	74	<i>mdh</i>
31	<i>mdh</i>	79	<i>mdh</i>
45	<i>mdh</i>	81-1	<i>mdh</i>
48-1	<i>mdh</i>	82	<i>mdh</i>
52	<i>mdh</i>	84-1	<i>mdh</i>
54	<i>mdh</i>	84-2	<i>mdh</i>
55	<i>mdh</i>	85	<i>mdh</i>
57-1	<i>mdh</i>	95	<i>mdh</i>
60	<i>mdh</i>	20	<i>mdh</i>
61	<i>mdh</i>	36	No bands detected
62	<i>mdh</i>	41	No bands detected
63	<i>mdh</i>		

**Figure 6.3** Multiplex PCR gel with products of the standard culture mix (SCM), *E. coli* ATCC and pathotype reference strains (ATCC 13135 – EAGG) and *E. coli* isolates (3-1, 64-1, 19, 82, 84-1, 85). Lanes “L” and “C” show a 100 bp ladder and negative control, respectively

All the 34 isolates phenotypically identified as *E. coli* were positive for *mdh*. Thirty-three of these isolates exhibited no pathotype bands and were considered members of the commensal *E. coli* grouping.

The *eagg* gene was detected in only one isolate (3-1) by the multiplex PCR. This isolate was isolated from water obtained from the Berg River (site Berg 1, refer to Figure 3.1) in September of 2008, and is considered as a member of the EAEC pathotype. The EAEC pathotype has been associated with developing countries where persistent diarrhoea in infants and children (Morelli *et al.*, 1994) and chronic diarrhoea in human immunodeficiency virus (HIV) patients (Moses *et al.*, 2006) routinely occur. This pathotype is considered an emerging pathogen and has been implicated in several outbreaks globally (Carlos *et al.*, 2011). EAEC strains are characterised by their adhesion by bundle-forming fimbriae to HEp-2 and HeLa cells (Morelli *et al.*, 1994). It is interesting to note that this isolate was obtained from the site which had the lowest mean for *E. coli* during the 13-month sampling period (see Chapter 3), and also in a month when *E. coli* counts were particularly low. This phenomenon indicates that the presence of *E. coli* pathotypes is not necessarily dependent on a high level of total *E. coli*. It is important to keep this in mind when risk assessments are conducted, since normal enumeration methods for *E. coli* do not take into account the proportion of pathogenic *E. coli*. This could possibly lead to a risk assessment overlooking a considerable potential health risk based on quantitative information alone.

The work reported by Carlos and co-workers (2011), was aimed at identifying reservoirs of *E. coli* pathotypes in the São Paulo area of Brazil. They found EAEC only among isolates from healthy humans and sewage samples. The presence of EAEC was not detected in any isolates from animals or water, and they concluded that healthy humans may act as carriers of this pathotype. The non-detection of EAEC in river water sampled in this study should, however, be addressed: testing for *eae*, *stx1* and *stx2* also resulted in non-detection from the river water, despite the fact that these genes were found in nearly all other potential reservoirs. However, Carlos *et al.* (2011) did state in their article that the river sampling site was situated in an environmentally protected area, and that only low levels of faecal pollution were expected. It is possible; therefore, that EAEC strains present in sewage simply never reached this part of the river and were, consequently, not detected. If the conclusions drawn by Carlos and co-workers are correct, the presence of EAEC in a water sample could implicate human faecal pollution.

Isolates 36 (*Enterobacter asburiae*) and 41 (*Enterobacter cloacae*), were included in the multiplex PCR as they both possessed the *tuf* gene. It was also found that both these organisms did not possess the *mdh* gene, and this, in conjunction with the absence of *uidA*, confirms that they do not belong to *E. coli*. In addition, the lack of these two specific *E. coli* genes also proves that the presence of the *tuf* gene in these organisms was not due to co-culture with strains of *E. coli*. This result is vindication for the API 20E and MALDI-TOF MS identifications, but raises doubt regarding the specificity of the *tuf* primer set. These primers were developed by Maheux *et al.*

(2009) specifically to detect a highly conserved region of the *E. coli tuf* gene. During specificity determinations done in this study, the *tuf* primers were tested on 139 species of Gram negative bacteria associated with faecal environments, including three strains of *Enterobacter asburiae* and two strains of *Enterobacter cloacae*. A high specificity (99.5% of all organisms tested) was found by Maheux *et al.* (2009) with the *tuf* primers, with only *Escherichia fergusonii* giving false positive reactions. However, *tuf* mRNA-DNA hybridisation experiments done by Filer & Furano (1980) showed 59% homology between *E. coli* and *Aerobacter* (now *Enterobacter*) *aerogenes*, primarily due to homology of the COOH-terminal of the gene. These workers suggested that this phenomenon could either be ascribed to horizontal gene transfer or to strong selective pressure in prokaryotes to retain segments of the *tuf* gene. In addition, the sequence of the *tuf* gene, which encodes for the EF-Tu elongation factor essential for bacterial translation, in *E. coli*, has considerable homology with the *tuf* genes from phylogenetically remote bacteria (Sela *et al.*, 1989). If this is the case, it is possible that the *tuf* PCR is not as specific as was reported by Maheux *et al.* (2009).

Isolate 20, which was identified as *Klebsiella pneumoniae* spp. *pneumoniae*, showed the presence of both the *uidA* and the *tuf* gene and was also positive for *mdh*. These results are surprising, since this organism gave high identification values for both API 20E and MALDI-TOF MS. Additionally, this organism grew and produced gas in EC with MUG, and showed the presence of three genes used in the identification of *E. coli*. Since *Klebsiella pneumoniae* does not possess a close homolog for the *uidRABC* operon in *E. coli* (McClelland *et al.*, 2000), and *uidA* was identified in the genome of isolate 20, it must be concluded that this isolate is a unique strain of *E. coli* which presents biochemical reactions more distinctive of *Klebsiella pneumoniae*.

## 6.5. CONCLUSIONS

### 6.5.1. Identity confirmation and pathotype grouping

In this study, the *lacZ* PCR showed unsatisfactory results for the detection of coliforms with primer annealing at 59.7°C. A variety of species within the coliform grouping could not be detected by this PCR analysis. The work of Bej *et al.* (1990) confirmed these findings, and they indicated that primer annealing at a decreased temperature (40°C) alleviated this problem in their study. If additional resources were available for method development, investigating the detection of these coliform isolates at various annealing temperatures may have produced similar results. This should be kept in mind for future work relating to the detection of coliforms which were originally isolated from river water.

The *uidA* and *tuf* monoplex PCR analyses were conducted to confirm the identity of isolates obtained from MTF steps. The molecular identification confirmed the *E. coli* identity in all the *E. coli* isolates. The confirmation of non-*E. coli* isolates was positive for 96.1% of non-*E. coli* isolates,

with two exceptions. The monoplex PCR results and phenotypic identifications reciprocally confirm the reliability of both the phenotypic and molecular approach to the identification of these isolates, with the exception of the *tuf* PCR of isolates 36 and 41. The identification of these two strains of *tuf* positive *Enterobacter*, along with reports in literature citing considerable homology between *E. coli* and *Enterobacter* (Filer & Furano, 1980), highlights the necessity to determine additional limitations of the *tuf* PCR. This should be done through a *tuf* PCR screening of a wide variety of *Enterobacter* strains from various environmental and clinical environments to determine the extent of the non-specificity of the primer set.

The multiplex PCR analysis showed that one out of the 34 *E. coli* strains was positive as an *E. coli* pathotype and was identified as an enteroaggregative strain. It is possible that the detection of such a low proportion of *E. coli* pathotypes was due to the loss of virulence factors. This phenomenon has already been described by Fagan *et al.* (1999), who reported the loss of *stx* genes after prolonged subculturing.

#### 6.5.2. Impact of molecular identification on MTF accuracy

The effect of molecular identification of the isolates on the distribution of atypical and typical organisms was shown in Table 6.16 to have a small impact on the accuracy of MTF as determined by phenotypic identification. The identification of a unique strain of *E. coli* (isolate 20) did result in a small increase in the number of false negative *E. coli*. If isolates of this nature are abundant in a given water source, this effect will be amplified and can result in a considerable under-estimation of *E. coli* numbers.

The detection of the *tuf* gene in two isolates of the genus *Enterobacter* in isolation may have lead to an incorrect re-designation of these isolates as *E. coli*. This would have increased the number of false positive coliforms (from isolate 41) and *E. coli* (from isolate 36 and 41), which in turn would have resulted in an incorrect over-estimation by MTF. These findings highlight the necessity for additional analyses when *tuf* is used as an indication of *E. coli* presence.

Another problematic observation emerging from this work is the non-detection of *uidA* in the genome of seven “false positive” *E. coli* isolates from EC broth with MUG. These isolates were initially isolated from MTF reactions where they caused, among others, a fluorescent reaction with MUG. The absence of *uidA* in their genome indicates that these isolates may possess another enzyme capable of hydrolysing MUG. However, it is more likely that they were co-occurring with another organism which did possess  $\beta$ -D-glucuronidase. If the latter is the case, these isolates are not true “false positives” and cannot, in isolation, result in false positive reactions.

#### 6.5.3. Implications of molecular identification and pathotype grouping for food safety

The impact of the identity of isolates on the food safety of produce irrigated with water from which they originate did not increase *per se* due to the results of the genotypic evaluation. The confirmation of the identity of *E. coli* isolates, as well as the identification of an additional isolate



(20) of *E. coli*, only serve to reassert the evidence of faecal contamination in the river water and the risk of transfer of enteric pathogens from river water to irrigated produce.

The identification of one enteroaggregative strain of *E. coli* among the isolates found in the Berg River does, however, greatly increase the potential risk of using this water for the irrigation of minimally processed foods. Since the isolation of the organisms was done using approaches and criteria for eventually determining method accuracy, the proportion of these pathotypes in the water cannot be calculated, or even estimated. However, it is highly probable that the *in situ* proportion of these pathotypes is much higher than it appears in this work (one pathotype from 35 *E. coli* isolates), since organisms were tested only after numerous purification steps which could have resulted in loss of virulence genes. In addition, the presence of this EAEC strain in the water is indicative of at least one infected individual, symptomatic or asymptomatic, higher upstream, who is likely to spread the infection in his/her community through the oral-faecal or foodborne route. Finally, bearing in mind the traditional role of *E. coli* as indicator of faecal pollution, the presence of EAEC in the Berg River could allude to the presence of a variety of other faecally-associated bacteria. If these organisms are transferred onto fresh produce through irrigation activities, the implications could be extremely detrimental for food safety in this country and its food export destinations.

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## CHAPTER 7

### NUMERICAL ANALYSIS OF ATYPICAL AND TYPICAL MULTIPLE TUBE FERMENTATION ISOLATES BASED ON BIOCHEMICAL REACTIONS AND RIBOSOMAL PROTEINS

#### 7.1. ABSTRACT

The observation that multiple tube fermentation (MTF) isolates identified as the same species through phenotypic and, in some cases, molecular techniques did not necessarily result in the same MTF reaction was the basis of this numerical analysis. Twenty-six biochemical characteristics determined by Analytical Profile Index (API) 20E and *E. coli*/coliform chromogenic agar were used to construct dendrograms of numerical relatedness of all MTF isolates using Jaccard and Sokal & Michener coefficients. A minimum of 20 ribosomal protein spectra were used to create main spectral projections (MSP) of MTF isolates identified as *E. coli*, using a technology based on matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). The MSPs of the 34 *E. coli* isolates were then utilised in the construction of a dendrogram through the transformation of unweighted average distances to dissimilarity values. These analyses were done to determine whether the biochemical and spectral information could be used to differentiate the same species of isolates which resulted in divergent MTF reactions, and thereby identify the problematic reaction or ribosomal protein responsible for the differences. The results of the numerical analysis based on biochemical reactions indicated that the combined characteristics determined through API 20E and chromogenic agar could not explain the different MTF reactions by the same strains. Isolates giving typical and atypical reactions were distributed in an interspersed manner along the dendrograms and no clustering of isolates with similar reactions occurred. Similarly, numerical analysis of the spectral data of the *E. coli* isolates showed that this information could not be used to differentiate false negative from true positive *E. coli* strains since no clustering of isolates eliciting that same MTF reaction occurred on the dendrogram. Although this work concluded that both API 20E-obtained biochemical reactions and ribosomal protein spectra were inappropriate predictors of MTF reactions, two interesting observations emerged. Firstly, the hydrolysis of 2-nitrophenyl- $\beta$ -D-galactopyranoside, tested as part of the API 20E test, could not effectively predict gas production from lactose in MTF. Secondly, it was observed that anaerogenic isolates could ferment a wider variety of carbohydrates in API 20E when compared to aerogenic isolates, a phenomenon which may indicate that anaerogenic isolates utilise these carbohydrates preferentially over lactose.

## 7.2. INTRODUCTION

When highly accurate enumeration results for coliforms and *E. coli* are required, MTF remains one of the important methods in modern microbiology. This is attributable to the selective hurdles included in the method, which excludes non-target organisms from the enumeration, as well as the semi-statistical quantification done through the use of most probable number (MPN) tables (Christensen *et al.*, 2002). This method or membrane filtration is prescribed by the South African Water Quality guidelines for the analysis of irrigation water (DWAF, 1996). For these reasons it was also used in the baseline microbiological monitoring of the Berg River, reported in Chapter 3, which was done as part of the WRC project K5/1773.

Despite these attributes of the method, the isolation and identification of isolates from MTF steps, done in Chapter 5, showed considerable variation in the MTF reactions of isolates which were identified as the same species. These results were confirmed in Chapter 8 (section 8.4.1), where isolates were tested individually to determine their true contribution to MTF reactions. This heterogeneity is presumably attributable to the environmental stresses which these bacteria were subjected to while in the river environment (Kivisaar, 2003). Such stress factors could have included a lack of carbon, nitrogen, phosphorous and oxygen, or may have been due to water pH or temperatures which were not optimal for sustaining these bacteria (Kivisaar, 2003). The latter two factors were examined in a previous chapter (see section 3.3.4), and the pH of the Berg River was indeed found to be extremely low in some instances during the 13 month sampling period. The identification of such isolates which have undergone adaptation, due to environmental or other stresses, to result in atypical or rare strains can be extremely difficult when using commonly available tests (Baron, 1996) such as the API system.

Despite these drawbacks, the numerical relatedness of organisms based on their phenotypic characteristics can offer a great deal of “hidden” information. For instance, the API 20E test consists of 22 characteristics, which are used for identification of unknown enteric bacteria. If these characteristics, which differ for each organism, are used to determine the relatedness of the organisms, a visual representation of their similarity or dissimilarity could be constructed. Such a visual representation could be extremely useful for determining whether groups of organisms causing the same reactions in MTF media are phenotypically similar to one another, and, importantly, different from other groups of organisms causing different reactions in MTF media. If this is the case, the “discrepant” phenotypic characteristics could be implicated in the discrepancy in reactions. The same applies to the ribosomal proteins of these organisms, where MALDI-TOF MS is applied to organisms to determine their ribosomal protein spectra for the construction of dissimilarity dendrograms. If the ribosomal protein composition of isolates with discrepant reactions in MTF media are found to differ from that of isolates which behave typically in the MTF, these compositional differences could be used to explain, at least in part, the differences observed in MTF media.

Consequently, the aim of this chapter was to construct dendrograms based on biochemical reactions and ribosomal protein composition of MTF isolates. This was done to elucidate whether numerical data could be used to differentiate organisms which caused atypical reactions in MTF from strains of the same organism which behaved typically.

**Note:** during the reporting of these results, the terms “aerogenic” and “anaerogenic” have been used for the description of isolates. In this context, this does not refer to the oxygen requirements of the organism, but the ability or inability to produce gas from lactose media.

### 7.3. MATERIALS AND METHODS

The construction of dendrograms showing the relatedness of atypical and typical organisms from the MTF method was done using 111 MTF isolates which had been previously characterised in terms of their biochemical reactions (see sections 5.4.3 to 5.4.6) and ribosomal proteins (see sections 5.4.9 to 5.4.12). Four American Type Culture Collection (ATCC) reference strains of *E. coli* (ATCC 11775, ATCC 4350, ATCC 10799 and ATCC 13135), which had also been characterised in terms of biochemical reactions and ribosomal proteins (see sections 5.4.2 and 5.4.8, respectively) were used as controls.

#### 7.3.1. Numerical analysis of isolates

Numerical relatedness based on biochemical reactions

In previous characterisation work, done in Chapter 5, on the 111 MTF isolates as well as four reference strains of *E. coli*, biochemical identification with API 20E (bioMérieux SA, Marcy l'Étoile, Rhône, France) was performed through determination of the utilisation of various compounds. These reactions were noted as positive or negative. The results of these tests were recorded according to the API convention, which awards a score to each individual positive reaction. The reactions are added in groups of three to obtain a code which is used to identify the organisms with the *apiweb* software (bioMérieux SA, Marcy l'Étoile, Rhône, France). The chemical compounds used and its purpose in the test, as well as the score awarded for each, are shown in Table 7.1.

**Table 7.1** Compounds and corresponding test reaction used in API 20E

Compound	Test for	Score	Compound	Test for	Score
2-nitrophenyl-β-D-galactopyranoside	β-galactosidase	1	D-mannitol	Fermentation/oxidation	1
L-arginine	Arginine dehydrolase	2	Inositol	Fermentation/oxidation	2
L-lysine	Lysine decarboxylase	4	D-sorbitol	Fermentation/oxidation	4
<b>Table 7.1 continued</b>					



**Table 7.1 continued**

Compound	Test for	Score	Compound	Test for	Score
L-ornithine	Ornithine decarboxylase	1	L-rhamnose	Fermentation/oxidation	1
Trisodium citrate	Citrate utilization	2	D-saccharose	Fermentation/oxidation	2
Sodium thiosulphate	H <sub>2</sub> S production	4	D-melibiose	Fermentation/oxidation	4
Urea	Urease	1	Amygdalin	Fermentation/oxidation	1
L-tryptophane	Tryptophane deaminase	2	L-arabinose	Fermentation/oxidation	2
L-tryptophane	Indole production	4	Oxidase (additional)	Cytochrome oxidase	4
Sodium pyruvate	Acetoin production	1	Nitrate reduction	NO <sub>2</sub> production	1
Bovine gelatine	Gelatinase	2	Nitrate reduction with zinc	Reduction of N <sub>2</sub>	2
D-glucose	Fermentation/oxidation	4			

The numerical clustering of biochemical characteristics and colony colour on *E. coli*/coliform chromogenic agar (Oxoid, Basingstoke, Hampshire, UK) was done using 111 isolates composed of 35 isolates from LST broth, 22 from BGLB broth, 41 from EC broth with MUG and 13 from L-EMB agar, as well as four *E. coli* reference strains. Twenty-six characters were included in the data set and analysed using the Jaccard (SJ) and Sokal and Michener (SM) coefficients and the unsorted similarity matrix was rearranged into groups by average linkage cluster analysis (Lockhart & Liston, 1970). Tests that gave uniform results for all the strains were excluded from the numerical analyses. Dendrogram distances were calculated based on the phenotypic characteristics as calculation concept. These dendrograms would give a visual representation of the relatedness or similarity of these organisms, with the aim of ideally obtaining distinct clusters which separate true positives from false negatives and true negatives from false positives. If this is the case, the discrepant characteristics in organisms causing incorrect enumeration with MTF is hoped to give some indication of why these organisms do not behave normally.

#### Numerical dissimilarity based on ribosomal protein spectra

The isolates which were identified as *E. coli* by the Microflex LT were used to construct another dendrogram. For this, the MALDI Biotyper software was used to construct a dendrogram from the MSPs of the organisms.

The ribosomal protein spectra of the 111 isolates were determined according to the protocol as described under section 5.3.3 in Chapter 5. The only divergence in protocol was that



eight spots of the supernatant of each isolate were placed on the MSP 96 ground steel target plate. In addition, the analysis was done automatically by pre-programming the autoExecute function MBT\_autoX.axe instead of running comparisons with spectra in the Bruker database.

Three good-quality spectra were obtained from each of the eight spots per organism to yield a total of 24 spectra. Once spectra were obtained, smoothing and baseline subtraction were performed with the MBT\_Process method in the flexAnalysis software (Bruker Daltonik GmbH, Bremen, Germany). The 24 spectra were then inspected for any flatline or outlier spectra, such as a spectrum with remarkably large peak differences. In addition, mass deviation of peaks was not allowed to exceed 500 ppm at any point within the spectra. After unacceptable spectra were deleted, at least 20 spectra remained for the creation of the MSP of the organism. These spectra were selected simultaneously in the MALDI Biotyper software (Bruker Daltonik GmbH, Bremen, Germany), and the MSP was created.

For the creation of the dendrogram, the software initially performed hierarchical clustering on the MSPs from the *E. coli* isolates through linkage by unweighted average distance. This clustering was subsequently transformed to a dendrogram by transforming the similarity values in the hierarchical clustering to dissimilarity values, which were normalised to a maximal distance level of 1 000 (0 denoting minimum and 1 000 denoting maximum dissimilarity) (Monika Schneider, 2011, Bruker Daltonik GmbH, Bremen, Germany, personal communication, 2011).

The purpose of drawing up this MSP dendrogram was, firstly, to see how it would compare with the dendrogram for *E. coli* isolates based on their phenotypic characteristics. Secondly, and more importantly, it was hypothesised that large dissimilarities would be found between organisms resulting in true positives and those producing false negative reactions during *in situ* MTF of river water. In addition to *in situ* reactions the individual reactions of isolates, which were determined and are reported under section 8.4.1, were also considered during this evaluation. This hypothesis was evaluated to ascertain whether discrepancy in the ribosomal protein spectra of these distinct groups could, in some part, assist in explaining the diverse reactions caused by *E. coli* in the MTF method.

## **7.4. RESULTS AND DISCUSSION**

### **7.4.1. Determination of numerical relatedness of isolated organisms**

Numerical relatedness based on phenotypic results

The API 20E results of the organism, given in the convention described under section 7.3.1, are shown in Table 7.2.

**Table 7.2** API 20E codes obtained for all isolates

Isolate	Code								Isolate	Code							
1	1	1	4	4	5	4	2	1	56	3	3	0	5	5	7	3	1
2	5	2	1	5	7	7	3	2	57-1	5	1	4	4	5	7	2	1
3-1	5	1	4	4	5	5	2	1	57-2	3	3	0	5	7	7	3	1
3-2	1	2	1	5	7	7	3	2	59	5	2	1	5	7	7	3	1
4	7	0	4	7	1	2	2	1	60	5	1	4	4	5	7	2	1
5	3	3	4	5	7	7	3	1	61	5	3	4	4	5	7	3	1
6	5	1	4	4	5	7	2	1	62	5	0	4	4	5	5	2	1
7	5	2	1	5	7	7	3	1	63	7	1	4	4	5	7	3	1
8	5	1	6	4	5	7	2	1	64-1	5	1	4	4	5	7	2	1
9	5	2	1	5	7	7	3	1	64-2	3	3	0	5	3	7	3	2
11-1	3	6	0	5	5	5	3	1	65	5	1	4	4	5	7	2	1
11-2	0	1	7	4	0	0	0	2	66	7	1	4	4	5	7	2	1
12	7	3	7	7	7	3	3	1	67	7	1	4	4	5	7	2	1
13-1	7	0	4	4	5	7	2	1	68	5	1	4	4	5	7	2	1
13-2	0	2	2	5	0	0	0	1	69	3	3	0	5	5	2	3	2
14	0	2	6	4	0	0	0	1	70	3	3	0	5	5	2	3	2
15	1	0	0	7	1	5	3	2	71	3	3	0	5	5	2	3	2
16	5	0	4	4	5	5	2	1	72	3	3	0	5	5	6	3	2
17	3	3	0	5	5	0	3	2	73-1	5	1	4	5	5	5	3	1
18	5	1	4	4	5	7	2	1	73-2	5	0	4	4	1	4	2	1
19	5	0	4	4	5	5	2	1	73-3	0	3	3	7	0	0	0	2
20	5	2	4	4	7	7	3	1	74	7	1	4	4	5	7	2	1
21	2	0	0	6	0	0	0	1	75	5	2	0	5	7	7	3	1
22	0	1	7	4	0	0	0	2	79	5	1	4	4	5	5	2	1
23	0	2	7	4	2	1	0	1	80	1	2	0	4	5	7	2	1
24-1	3	7	3	7	5	7	3	1	81-1	7	0	4	4	5	5	2	1
24-2	0	1	7	4	0	0	0	2	81-2	1	2	0	4	7	7	3	2
25	3	3	0	5	5	7	3	2	82	7	0	4	4	5	5	2	1
26	1	0	0	0	0	0	0	2	84-1	5	1	4	4	5	1	2	1
27	0	0	0	6	0	0	0	1	84-2	5	1	4	4	5	5	2	1
28	1	0	0	4	5	5	3	2	85	5	0	4	4	5	4	2	1
29	5	2	5	4	7	7	3	1	86	5	2	4	5	7	7	3	0
30	7	3	5	7	7	2	3	1	87	3	1	0	5	5	6	3	1
31	7	1	4	4	5	7	2	1	88	3	3	0	5	5	2	3	2
32	2	2	0	2	0	0	0	2	89	5	3	1	7	7	2	0	1
34	3	3	0	5	5	7	3	2	90	5	2	0	5	7	7	3	1
35	5	3	0	7	7	2	1	1	92	1	0	4	4	5	7	3	1
36	3	3	0	5	7	2	3	2	93	3	1	0	5	5	6	3	1

**Table 7.2 continued**

Table 7.2 continued																	
Isolate		Code							Isolate		Code						
37	5	2	0	7	7	7	3	2	94	3	2	0	4	7	7	2	1
38	5	2	4	5	7	7	3	2	95	3	1	0	4	5	7	2	1
39	5	3	0	5	1	1	2	1	97	3	3	0	5	7	2	3	1
40	5	2	4	5	7	7	3	2	98	0	7	7	6	2	0	0	1
41	3	3	0	5	5	7	3	1	99	0	0	7	6	0	2	0	1
42	3	3	0	5	5	2	3	2	100	0	7	3	7	0	0	0	1
43	1	2	0	7	3	6	3	1	101	0	4	7	7	0	2	1	1
44	0	2	7	4	3	1	1	1	102	0	4	7	6	0	2	0	1
45	7	0	0	4	5	7	2	1	103	0	7	7	6	2	0	0	1
46	3	2	0	6	1	2	2	1	104	0	2	2	4	2	0	0	1
47	3	2	0	4	5	1	2	1	105	0	7	3	7	0	0	0	1
48-1	5	1	4	4	5	7	2	1	106	0	3	3	6	0	0	0	1
48-2	5	3	0	6	7	7	3	1	107	0	4	7	6	0	2	0	1
49	5	2	0	5	7	7	3	2	108	5	6	7	7	7	7	3	2
50	0	0	0	6	0	0	0	1	109	0	2	6	4	2	0	1	1
51	5	2	5	5	7	7	3	1	EC58	5	1	4	4	5	5	2	1
52	7	1	4	4	5	5	2	1	EC157	5	1	4	4	5	5	2	1
53	5	2	0	5	7	7	3	1	EC158	5	1	4	4	5	5	2	1
54	7	1	4	4	5	7	2	1	EC404	5	1	4	4	5	5	2	1
55	7	1	4	4	5	7	2	1									

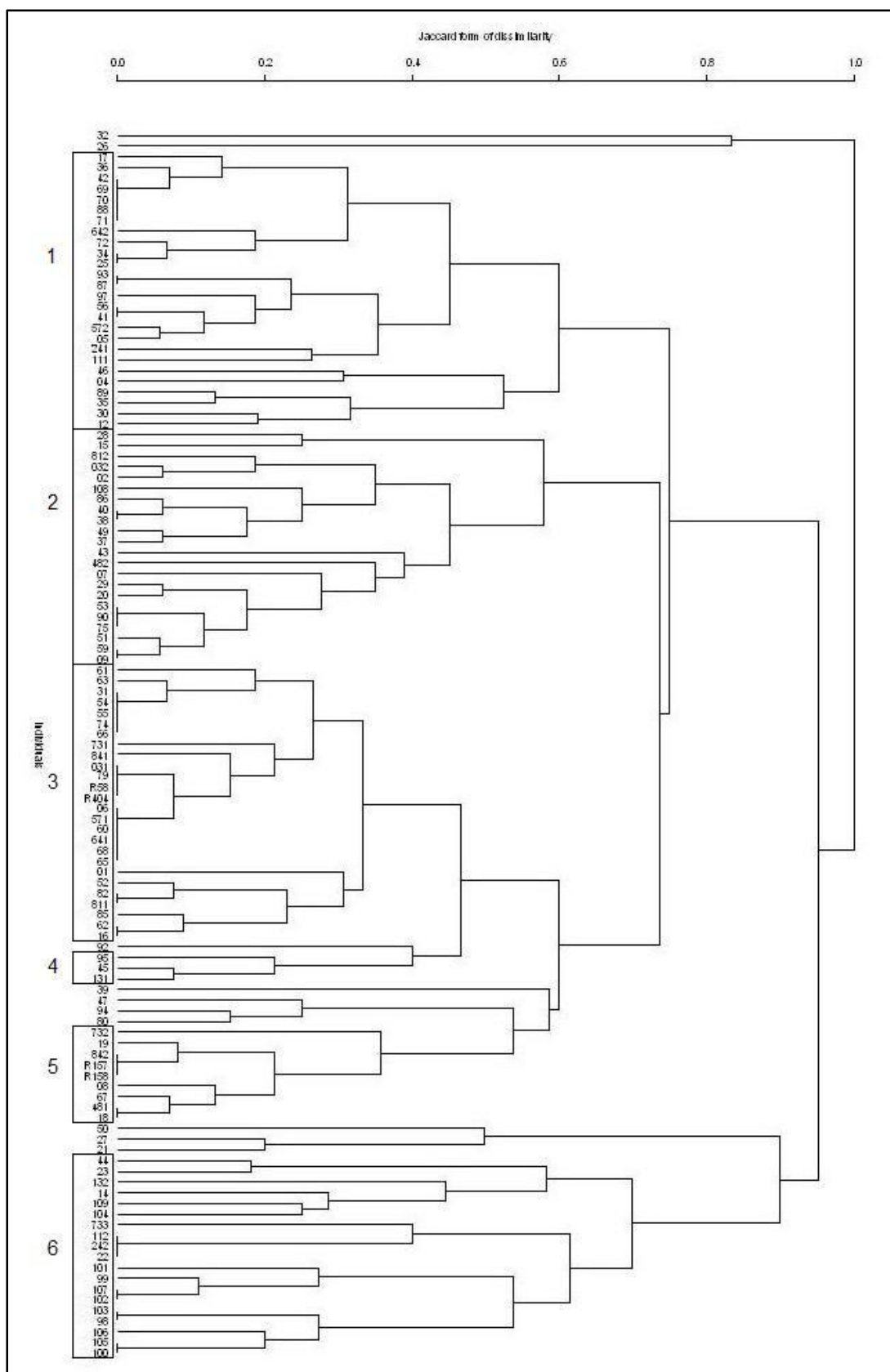
The results for the hydrolysis of 2-nitrophenyl- $\beta$ -D-galactopyranoside show an unexpected and interesting outcome. Of the 111 isolates tested, 21 could not hydrolyse this compound. Since the breakdown of galactopyranoside is catalysed by the enzyme  $\beta$ -D-galactosidase, which is associated with the hydrolysis of lactose into its monosaccharide components (Manafi *et al.*, 1991) by coliforms, a negative reaction for this test should be indicative of anaerogenesis in the presence of lactose. With closer examination, the 21 isolates having 0, 2, 4 or 6 in the first position of the API 20E code were found to be from both aerogenic and anaerogenic reactions. This could be indication that the hydrolysis of 2-nitrophenyl- $\beta$ -D-galactopyranoside is an inappropriate predictor of the ability to produce gas from lactose in MTF media. Alternatively, the identification of 2-nitrophenyl- $\beta$ -D-galactopyranoside negative organisms from aerogenic reactions may be due to the production of gas in the medium by an organism other than the one isolated, which highlights the limitations of the isolation sampling procedure.

One isolate (32) was obtained from L-EMB agar, and therefore no conclusion could be made for this organism since its *in situ* reaction in lactose broth is unknown. However, the individual MTF analysis performed in Chapter 8 showed that this organism produced a weak gas reaction in LST broth and no gas in EC broth with MUG, but could produce gas in BGLB broth. Therefore, it is accepted that this organism, although it is not strictly unable to ferment lactose,

does not ferment lactose readily. Eight (11-2, 14, 21, 22, 23, 24-2, 44 and 50) of the isolates which could not hydrolyse 2-nitrophenyl- $\beta$ -D-galactopyranoside were originally from anaerogenic reactions in MTF media. Their inability to hydrolyse this compound is, therefore, not surprising. Furthermore, one of these isolates (21) was identified by MALDI-TOF MS as *Micrococcus luteus*. Therefore, this isolate is not a coliform and is not expected to produce gas in the media. It is surprising to note, however, that these anaerogenic and 2-nitrophenyl- $\beta$ -D-galactopyranoside negative organisms only make up 15.1% of the total number of anaerogenic isolates. The large proportion of originally anaerogenic isolates which exhibited  $\beta$ -D-galactosidase activity with API 20E could confirm the findings of Edberg & Edberg (1988) and Evans *et al.* (1981). These workers attributed the anaerogenic reactions of aerogenic coliforms to the suppression of the organisms by indigenous bacteria found in the environmental sample being tested. Another point of interest is the observed  $\beta$ -D-galactosidase activity in API 20E by three non-coliforms (4, 26 and 37), which were originally isolated from anaerogenic reactions. The reason for their anomalous profiles is unclear, but it is possible that the inhibition by autochthonous bacteria suppressed their ability to produce gas from lactose, as with coliforms. Their positive test for  $\beta$ -D-galactosidase is troublesome, however, since it alludes to a low specificity of this test for detecting coliforms.

Furthermore, 11 of the isolates (98, 99, 100, 101, 102, 103, 104, 105, 106, 107 and 109) were obtained from MTF reactions where gas was produced from lactose in the initial reaction. It is possible that the true organism responsible for the aerogenic reaction in these cases was not isolated, since the isolation procedure takes the form of a grab sample. All these isolates were identified by MALDI-TOF MS as belonging either to the genus *Proteus* or *Providencia*, both of which form part of the coliform grouping. It is likely that these coliforms are not avid gas producers, but are particularly adept at growing during the sub-culturing procedures which were employed during the isolation. This would account for the aerogenesis in the medium, probably induced by another organism, and the contradictory 2-nitrophenyl- $\beta$ -D-galactopyranoside reaction. However, this cannot be proven with the experimental data presented here, since individual reactions of these isolates within the media of the MTF method would have to be determined.

The Jaccard dissimilarity dendrogram based on the phenotypic characteristics of all the isolated organisms, as well as four reference strains of *E. coli*, is given in Fig. 7.1. Hyphens were removed from numbers in the dendrogram but are used in text. The dendrogram in Fig. 7.1 indicates that two of the isolates (26 and 32) were maximally dissimilar from the rest of the isolates. These two isolates, identified by MALDI-TOF MS as *Bacillus pseudofirmus* and *Enterobacter asburiae*, were isolated from atypical reactions occurring in LST broth (isolate 26) and on L-EMB agar (isolate 32). Their large dissimilarity from the rest of the group is presumably due to their failure to utilise or hydrolyse most of the test compounds in the API 20E strip: isolate 26 could only hydrolyse 2-nitrophenyl- $\beta$ -D-galactopyranoside and produce  $N_2$  gas with the addition of zinc to the glucose reaction cupule; and isolate 32 could only elicit positive reactions for arginine dehydrolase, citrate utilisation, gelatine hydrolysis,  $N_2$  production with zinc, and grow as pink colonies.



**Figure 7.1** Jaccard form of dissimilarity based on phenotypic characteristics of all isolates (indicated categories 1-6 are based on clustered isolate identities)

There are a number of isolates which did not produce many positive reactions with API 20E. For instance, isolate 50 could only hydrolyse gelatine, ferment glucose, reduce nitrate to NO<sub>2</sub>, and grow as pink colonies on chromogenic agar. Isolates 21 and 27 behaved similarly, but could, in addition, produce a positive reaction for arginine dehydrolase and grew as white colonies on chromogenic agar. These three isolates did not, however, differ maximally from the rest of the group in Fig. 7.1, which discounts the possibility of many negative reactions causing the dissimilarity in isolates 26 and 32. Furthermore, isolates 26 and 32 were grouped together, they differed considerably (0.84) from each other as well. Although MALDI-TOF MS identified isolate 32 as *Enterobacter asburiae*, API 20E indicated that the organism belongs to *Pseudomonas aeruginosa* (99.9% identification). However, since the MALDI-TOF MS best match value was very high (2.344), the identification is most probably correct. The discrepancies of this isolate from the rest of the *Enterobacter* isolates may indicate that this organism is a rare atypical strain of *Enterobacter*. The largely discrepant biochemical profile is illustrated in Table 7.3, where the API codes for the 23 biochemical reactions of this isolate as well as three *Enterobacter* isolates, randomly selected from different areas in category 1, are given.

**Table 7.3** API 20E codes for isolate 32 and three other *Enterobacter* strains

Isolate	Code							
32	2	2	0	2	0	0	0	2
24-1	3	7	3	7	5	7	3	1
42	3	3	0	5	5	2	3	2
93	3	1	0	5	5	6	3	1

The groupings of the rest of the organisms were divided into six broad categories, which can be seen on Fig. 7.1. The first category is a grouping of *Citrobacter*, *Enterobacter* and *Serratia* isolates. The only exceptions within the group are the presence of isolate 4, 26 and 46, identified as *Aeromonas hydrophila*, *B. pseudofirmus* and *Klebsiella pneumoniae*, respectively. The isolates in this grouping are from atypical reactions of LST, BGLB, and L-EMB and both typical and atypical reactions from EC broth with MUG. To determine whether the biochemical characteristics had any bearing on the typical or atypical behaviour of organisms, the API 20E codes for the 23 reactions were compared in isolates 64-2, 69, 70, and 72 is shown in Table 7.4. These isolates were chosen since only EC broth with MUG was represented in this category by typical (64-2) and atypical (69, 70 and 72) reactions.

**Table 7.4** API 20E codes for six isolates obtained from EC with MUG

Isolate				Code				
64-2	3	3	0	5	3	7	3	2
69	3	3	0	5	5	2	3	2
70	3	3	0	5	5	2	3	2
72	3	3	0	5	5	6	3	2

It is evident from Table 7.4 that the codes differed very little. The codes for the isolates obtained from atypical reactions corresponded well with that of the isolate from a typical reaction. Codes for isolates 69 and 70 were identical to isolate 64-2, except for their additional ability to ferment D-sorbitol instead of inositol. Isolate 72, which failed to produce gas or fluorescence in EC broth with MUG, differed from the other atypical isolates only in its ability to ferment D-melibiose. It is highly unlikely that this ability could be associated with a failure to hydrolyse MUG and produce fluorescence, since it is well known that the hydrolysis of MUG is induced by the enzyme  $\beta$ -D-glucuronidase. Furthermore, the additional ability of this organism to ferment melibiose does not account for its failure to produce gas from lactose in EC broth, since the production of gas from lactose is indicated by a positive  $\beta$ -D-galactosidase reaction with 2-nitrophenyl- $\beta$ -D-galactopyranoside.

The three isolates from atypical EC broth with MUG reactions could all hydrolyse 2-nitrophenyl- $\beta$ -D-galactopyranoside. This is in direct contradiction with their anaerogenic reactions in the medium, which could be explained either by defective  $\beta$ -D-galactosidase which cannot catalyse the breakdown of lactose, suppression by indigenous bacteria in the sample or, more likely, non-functional components also involved in the fermentation of lactose. To this end, the work by Gest and Peck (1955) shows that the enzyme system responsible for the decomposition of formate (an intermediate product in the breakdown of pyruvate) to carbon dioxide and hydrogen gas requires the presence of functional formic dehydrogenase and hydrogenase as well as an electron carrier. If any of these components are not present in an organism, gas cannot be produced from disaccharides. Whatever the reason may be for the contradictory results, the biochemical characteristics which are reflected through the API 20E results would not have been able to predict the behaviour of the organisms in media of the MTF method.

The second category in the dendrogram is comprised in majority by *Klebsiella*, *Raoultella* and *Serratia* isolates. Interspersed therein are single isolates of *Bacillus* (37), *Enterobacter* (43) and *E. coli* (7). All four steps within the MTF method are represented within the category, with both typical and atypical reaction isolates for BGLB broth, EC broth with MUG and L-EMB agar. To determine the biochemical differences between the same organism causing both typical and atypical reactions in a given medium, four isolates of *Klebsiella* causing typical (49) and atypical (2,



3-2 and 81-2) colonies on L-EMB agar were selected. The API 20E codes obtained for these isolates are given in Table 7.5.

**Table 7.5** API 20E codes for four isolates obtained from EC with MUG

Isolate				Code				
49	5	2	0	5	7	7	3	2
2	5	2	1	5	7	7	3	2
3-2	1	2	1	5	7	7	3	2
81-2	1	2	0	4	7	7	3	2

As with the previous comparison, the codes did not differ radically from one another. There were some differences however, especially in the first and third positions. The code for isolate 2 corresponded very closely with that of isolate 49, and only differed in the possession of urease activity by isolate 2. Once again, it is unlikely that the lack of urease in isolate 49 explains the production of metallic green colonies by the organism, since the metallic green sheen is associated with the precipitation of methylene blue in high acid conditions induced by the fermentation of lactose.

The codes for the two remaining isolates originating from very similar atypical reactions, mucoid pink (3-2) and mucoid purple (81-2) colonies, differed only in the lack of urease and acetoin production in isolate 81-2. Furthermore, the main biochemical difference between these two organisms and isolate 2, which produced mucoid purple colonies identical to those of isolate 81-2, is the lack of arginine dehydrolase and lysine decarboxylase in 3-2 and 81-2. Since isolates 2 and 81-2 produced the same colonies even with this discrepancy, the lack of these enzymes are probably not responsible for the production of mucoid pink colonies by 3-2.

The presence of *E. coli* isolate 7 within category 2 is interesting. This organism was identified by API 20E as *Klebsiella pneumoniae* (97.7% identification) based on its biochemical reactions. The MALDI-TOF MS identification of the organism as *E. coli* was equally convincing, with the best match producing a value of 2.350. The most prominent differences between this isolate and the *E. coli* isolates in category 3, obtained from typical EC broth with MUG reactions, was a negative indole reaction and positive reactions for acetoin production and inositol fermentation. Since this organism was isolated from a tube of EC broth with MUG where growth and fluorescence was observed without gas, a biochemical predictor of this reaction would probably have manifested in a difference relating to a failure to ferment carbohydrates that other *E. coli* isolates could ferment, or the absence of  $\beta$ -D-galactosidase. This was not the case however, and strengthens the belief that these reactions cannot indicate the reactions of isolates within media of the MTF. In addition, the organism was tested in an exploratory study with *lacZ* primers and exhibited the amplicon, indicating that it is capable of producing  $\beta$ -D-galactosidase. The

considerable discrepancy in the biochemical profile of isolate 7 when compared to other strains of *E. coli* and especially its negative indole reaction, which is an important determinant during *E. coli* identification using Parr's IMViC test (Leclerc *et al.*, 2001), alludes to the possibility that isolate 7 is a rare, atypical strain of *E. coli*.

Finally, isolate 20, with its discrepant reactions with MALDI-TOF MS (*Klebsiella pneumoniae*) and PCR (presence of *uidA*, *tuf* and *mdh*) clustered within this *Klebsiella* category, due to its biochemical characteristics. This isolate did not differ greatly from the other *Klebsiella* isolates within the category, and its possession of *E. coli* gene fragments indicate that this isolate is either a novel strain of *K. pneumoniae* or that the transfer of genetic attributes had somehow occurred between this organism and a strain of *E. coli*.

Category 3 in Fig. 7.1 is the first and largest *E. coli* category of the dendrogram. It comprises only *E. coli* isolates, typical and atypical, from EC broth with MUG and L-EMB agar as well as two reference strains of *E. coli* (ATCC 11775 and ATCC 13135, shown in Fig. 7.1 as R58 and R404, respectively). The two groups contained in this category, 61 to 65 and 1 to 16, differ at a level of 0.34. An isolate from a typical L-EMB reaction (isolate 31) was compared with an isolate from the other group with an atypical L-EMB reaction (isolate 81-1). It was found that the only biochemical differences according to API 20E is that the typical isolate lacked ornithine decarboxylase and the ability to ferment D-saccharose, a disaccharide similar to lactose. The failure of isolate 31 to ferment this carbohydrate, therefore, could possibly indicate that the organism favours the utilisation of lactose over that of sucrose. To verify this possibility, the sugar utilisation reactions of the other typical L-EMB isolate in this category, isolate 52, was investigated. This isolate was found to be unable to ferment only three of the carbohydrates in the test: amygdalin, inositol and D-saccharose. However, the three other isolates with atypical L-EMB reactions (1, 3-1 and 16) were also unable to ferment D-saccharose, discounting the theory that an inability to ferment saccharose is related to an ability to ferment lactose and, consequently, produce metallic green colonies. Additionally, one of these isolates (3-1) was identical to the two previously mentioned reference strains of *E. coli*, which produced metallic green colonies on L-EMB agar.

Only one *E. coli* isolate (6) from an atypical tube of EC broth with MUG was found in this category. This isolate occurred in an identical grouping with five isolates (57-1, 60, 64-1, 65 and 68) from typical EC broth with MUG reactions. The absence of any biochemical differences between these isolates and isolate 6, which produced an anaerogenic reaction in EC broth with MUG, confirms the conclusion that the API 20E results cannot be used to predict or explain the reactions observed in the MTF method.

Categories 4 and 5 were smaller clusters of *E. coli* isolates. Category 4 was comprised of three isolates which elicited atypical reactions in LST broth or L-EMB agar. Category 5 contained isolates resulting in typical and atypical reactions in EC broth with MUG, one isolate which caused an atypical reaction in LST broth, the remaining two reference strains of *E. coli* (R157 and R158),

and, interestingly, a strain of *K. pneumoniae*. The differences between isolates in this category were small, with the largest dissimilarity at 0.36. When two isolates (19 and 73-2) dissimilar on this level were compared, it was found that the only difference between the two was the ability of isolate 19 to ferment D-sorbitol and L-rhamnose. The atypical reaction of isolate 19 in EC broth with MUG was the failure to produce fluorescence, which is attributable to the lack of  $\beta$ -D-glucuronidase in the organism. Therefore, the biochemical differences could not be used to predict the lack of fluorescent reaction by the organism.

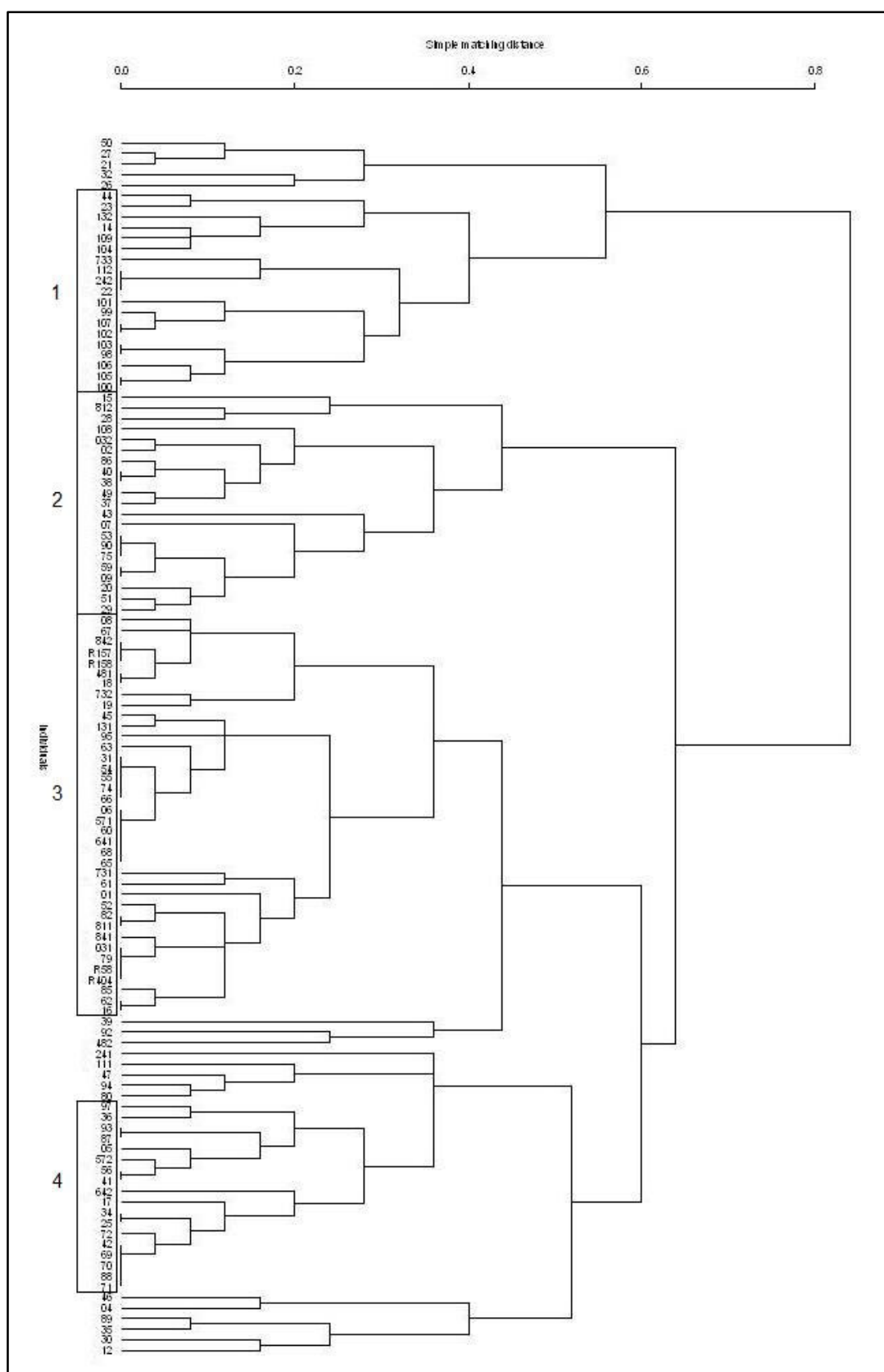
The presence of the *K. pneumoniae* isolate (8) in category 5 is due to its initial identification by API 20E as *E. coli* (99.4% identification). The results by MALDI-TOF MS were, however, equally compelling. This identification by ribosomal protein spectrum produced a best match value of 2.474. Its anaerogenic reaction, paired with an inability to produce fluorescence, in EC broth with MUG is indicative of some anomalous reaction by this strain. These reactions in combination with its clustering with *E. coli* isolates, alludes to the possibility that this is a very rare environmental strain in the genus *Klebsiella*. Alternatively, it may also be an atypical strain of *E. coli* with atypical MTF reactions.

Finally, category 6 comprised isolates from the genera *Morganella*, *Proteus* and *Providencia*, which were isolated most frequently from LST and BGLB broths. All isolates from LST broth were obtained from atypical reactions, and isolates from BGLB broth were found in both typical and atypical reactions. One isolate (73-3) was obtained from a typical reaction in EC broth with MUG. Larger dissimilarities, when compared to category 5, were observed in this category: the highest dissimilarity was at the 0.7 level, which divides the category roughly in half. No isolates from the same genera which caused typical and atypical reactions in LST or BGLB broth could be found. Instead, it appears from this dendrogram that isolates from the genus *Morganella* tended to produce atypical reactions in both broths, those from *Proteus* produced only typical reactions in both, and those from *Providencia* resulted in only typical reactions in BGLB but mostly atypical reactions in LST broth. However, since anaerogenesis is the only atypical reaction described in these two broths, a typical (13-2) and atypical (44) isolate belonging to the genus *Providencia* were selected for comparison. When examining the differences by API 20E, it was found that the atypical isolate possessed the ability to produce positive urease and indole reactions and could ferment mannitol, D-sorbitol, L-rhamnose, amygdalin and inositol, but could not produce acetoin from sodium pyruvate. The ability to ferment additional carbohydrates is counter-intuitive to use as predictor of anaerogenesis, but it is interesting to observe that many of the anaerogenic isolates discussed in this section had the ability to ferment more carbohydrates when compared to their aerogenic counterparts. The evidence is insufficient, however, to make such a conclusion from these findings. Furthermore, the lack of  $\beta$ -D-galactosidase activity in these two species of *Providencia* strengthens the belief that organisms from this genus are not particularly proficient at producing gas from lactose in the media of the MTF method.

The results discussed here, on the contrary, provides a great deal of evidence to support the contention that these biochemical properties are not appropriate to predict or explain the behaviour of an isolate in the MTF method. In addition, the interspersed arrangement of organisms from typical and atypical reactions within this dendrogram also indicates that these characteristics are not the determinants of typical or atypical behaviour.

The Sokal and Michener simple matching dendrogram based on the phenotypic characteristics of all the isolated organisms, as well as four reference strains of *E. coli*, is given in Fig. 7.2. Hyphens were removed from numbers in the dendrogram but are used in text. The first category (1) in the dendrogram (on the next page) is identical to category 6 in the Jaccard dissimilarity dendrogram. The category is composed entirely of isolates identified as *Morganella*, *Proteus* and *Providencia*, obtained from LST and BGLB broths. One exception was the same isolate (73-3) found in category 6 on the Jaccard dendrogram, which was isolated from EC broth with MUG. The only difference is that the highest level of dissimilarity between the isolates was 0.4 in this case.

The second category in Fig. 7.2 represents the grouping of isolates from the genera *Klebsiella* and *Raoultella*. Unlike the *Klebsiella* category in the Jaccard dissimilarity form *Serratia* isolates did not fall in this group but were grouped, together with single isolates of *Aeromonas*, *Klebsiella* as well as *Citrobacter*, underneath category 4 in Fig 7.2. The *E. coli* isolate (7) which clustered with *Klebsiella* isolates in the Jaccard dendrogram was grouped with these isolates once again, emphasising its anomalous biochemical profile and reaffirming the possibility that this organism is an atypical strain of *E. coli*. Since the statistics employed during the construction of the simple matching dendrogram differs from that used for the Jaccard dissimilarity form, the *Klebsiella* isolate (46) which diverged from the *Klebsiella* category was compared with a *Klebsiella* isolate (15) which remained in the category. The dissimilarity between these two organisms was at a level of 0.54, and it when the codes for these organisms were examined it was found that isolate 15 differed from 46 in its ability to ferment amygdalin, L-rhamnose, D-melibiose, but not D-saccharose. Furthermore, it had the ability to produce acetoin, but could not utilise citrate and does not possess arginine dehydrolase. Therefore, although both of these isolates were obtained from LST broth with atypical reactions, they exhibited extremely diverse reactions with API 20E. This is further evidence in support of the observation that these biochemical reactions cannot predict the reactions of organisms in media of the MTF.

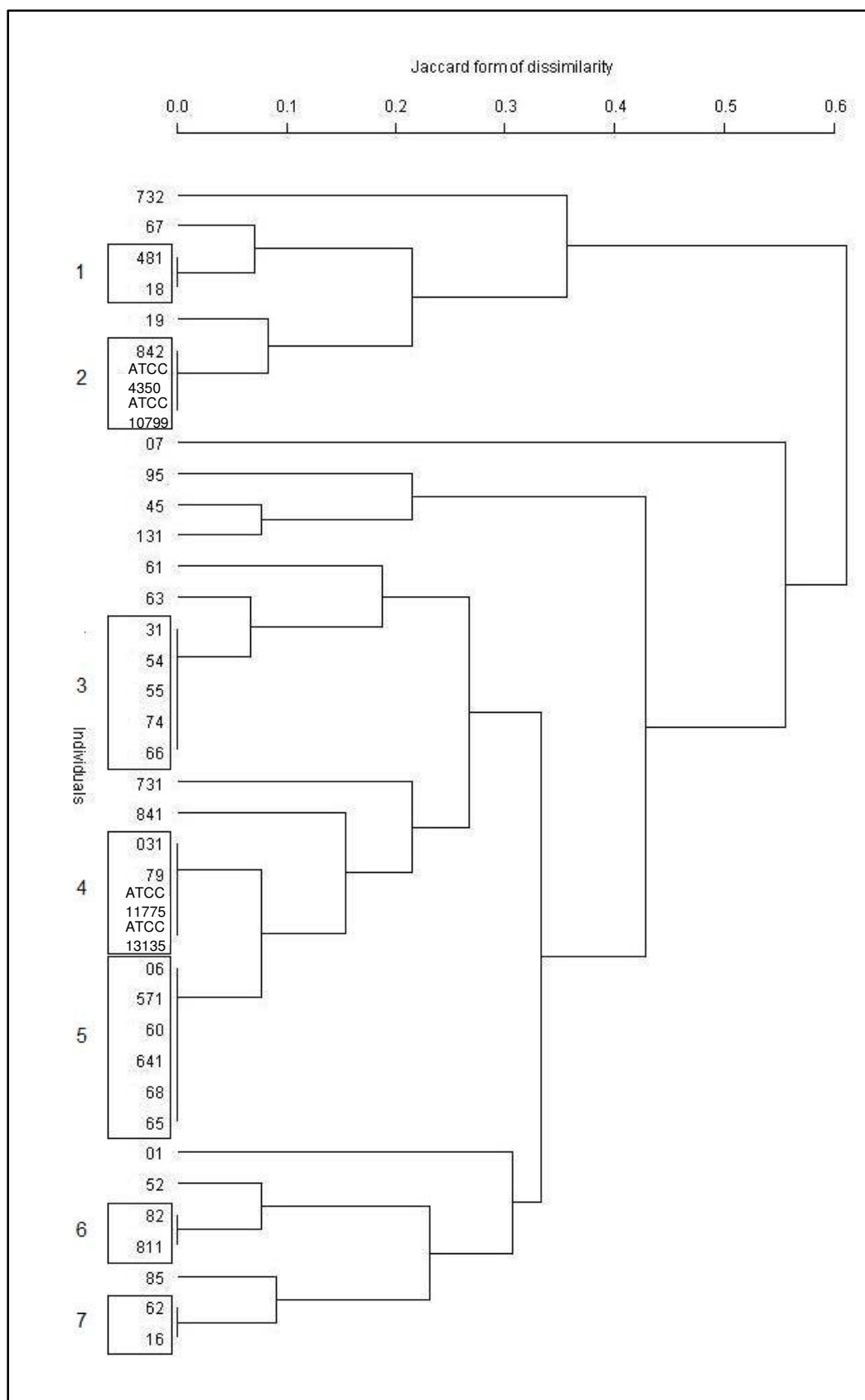


**Figure 7.2** Sokal and Michener simple matching dendrogram based on phenotypic characteristics of all isolates (indicated categories 1-4 are based on clustered isolate identities)

In the Sokal and Michener dendrogram in Fig. 7.2, all the *E. coli* isolates grouped together in one category, marked as category 3. This category contained all four reference strains of *E. coli* as well as isolate 8. This isolate, identified as *Klebsiella pneumoniae*, differed from the most dissimilar *E. coli* isolates on a 0.37 level. Its identification by API 20E was *E. coli* (99.4%), which indicates that this may be a unique strain of *K. pneumoniae* with many *E. coli* attributes. The biochemical characteristics of two anaerogenic isolates of *E. coli*, obtained from LST (48-1) and EC broth with MUG (6), were compared to determine the differences between the two. These isolates differed only in their chromogenic reactions: isolate 48-1 produced pink colonies on chromogenic agar, and isolate 6 produced purple colonies. In this case, therefore, their dissimilarity at the 0.37 level could only be ascribed to the difference in chromogenic reaction. In addition, isolate 6 showed no dissimilarity (100.0% similarity) with five other strains of *E. coli* (57-1, 60, 64-1, 65 and 68), which resulted in typical reactions in EC broth with MUG. Therefore, by extension, there is no difference biochemically between these typical isolates and the atypical isolate 48-1, except for their reactions on chromogenic agar.

The fourth category in Fig. 7.2 is a group of *Enterobacter* isolates similar to category 1 in the Jaccard dissimilarity form. The highest level of dissimilarity in this group is 0.28. When two *Enterobacter* isolates which differed at this level were compared, the difference between an isolate with typical reactions in EC broth with MUG (70) and one with an anaerogenic atypical reaction in EC broth with MUG (97) was only that isolate 97 could ferment inositol and reduced nitrate to  $\text{NO}_2$  and not  $\text{N}_2$ , as was the case with isolate 70. These differences cannot explain the difference in ability to ferment lactose. However, the tendency of anaerogenic strains to be capable of fermenting a greater variety of carbohydrates when compared to aerogenic counterparts is once again observed, and may be considered a predictor of anaerogenesis if extensive empirical evidence could be obtained to support this phenomenon. In broad terms, however, and as discussed previously, the differences based on API 20E observed between organisms cannot account for the differences in the reactions they elicit. Therefore, these characteristics are extremely inappropriate predictors of behaviour in the MTF method.

The Jaccard dissimilarity dendrogram constructed with the results of only the isolates identified by API 20E or MALDI-TOF MS as *E. coli* is given in Fig. 7.3. Hyphens were removed from numbers in the dendrogram but are used in text. Of the 38 *E. coli* organisms, both isolates and reference strains, used to construct the dendrogram in Fig. 7.3, only three had API 20E identification percentages below 98.6. These isolates were identified as *E. coli*, but the identification value was only 89.7% for isolate 1, 50.0% for isolate 63 and 63.8% for isolate 73-1. Furthermore, three of the isolates were identified as non-*E. coli* by API 20E, and were only included after their identification by MALDI-TOF MS. These isolates are 7, 61 and 95 and were identified as *Klebsiella pneumoniae* spp. *pneumoniae* (97.7%), *Kluyvera* spp. (98.3%) and *Enterobacter cloacae* (64.4%) by API 20E.



**Figure 7.3** Jaccard form of dissimilarity based on the phenotypic characteristics of isolates identified as *E. coli* and four *E. coli* reference strains



The MALDI-TOF MS identification of these isolates as *E. coli* were very high, however, with isolates 7, 61 and 95 producing best match values of 2.350, 2.476 and 2.406, respectively. The highest level of difference between the isolates in the entire group (n=38) was 0.6, and when the API 20E biochemical profiles of the two of the isolates (73-2 and 62) dissimilar on this level were compared, it was found that the only difference was that isolate 62 had the ability to ferment D-sorbitol and L-rhamnose and grew as purple colonies on chromogenic agar, as opposed to the pink colonies produced by isolate 73-2. Therefore, there is a large degree of biochemical similarity for the analysed characteristics in this group despite varied reactions, including both typical and atypical reactions in EC with MUG and on L-EMB agar as well as atypical reactions in LST broth.

There were seven clusters of several isolates or pairs of isolates which were 100.0% similar. The first cluster comprised of an anaerogenic strain from LST broth, and a non-fluorescing strain from EC broth with MUG. The second clustered an isolate from a typical EC broth with MUG reaction together with two reference strains (ATCC 4350 and ATCC 10799, shown in Fig. 7.3 as Ec157 and Ec158, respectively). The clustering of these two reference strains apart from reference strains ATCC 11775 (Ec58) and ATCC 13135 (Ec404), which was also observed in Fig 7.1 and 7.2, was due to their production of pink colonies on chromogenic agar. Similarly, reference strains ATCC 11775 (Ec58) and ATCC 13135 (Ec404) were again clustered together, due to their production of purple colonies on chromogenic agar. The third cluster with 100.0% similar organisms contained four isolates from typical EC broth with MUG reactions grouped with an isolate obtained from typical colonies on L-EMB agar. The fourth cluster comprised the aforementioned Ec58 and Ec404 strains together with an isolate from atypical L-EMB colonies and an isolate from a typical reaction in EC broth with MUG. The fifth, and most interesting, cluster was composed of five isolates obtained from typical EC broth with MUG reactions and one isolate from an atypical EC broth with MUG reaction. Cluster 6 and 7 both contain only two isolates, one from atypical colonies on L-EMB and one from a typical EC broth with MUG reaction.

As mentioned, cluster 5 was of particular interest for refuting the hypothesis that the biochemical characteristics investigated by API could be used to explain the variety of reactions observed by strains of the same species in the same medium. Isolate 6 elicited an anaerogenic reaction in EC broth with MUG, while the five other isolates produced perfect typical reactions in this medium. Since the six organisms were identical according to the biochemical properties tested for by API 20E, these characteristics did not detect the inability of isolate 6 to produce gas from lactose. The API 20E test which should have indicated this anomaly in isolate 6 was the  $\beta$ -galactosidase test, analysed for by 2-nitrophenyl- $\beta$ -D-galactopyranoside, since this enzyme is associated with the fermentation of lactose (Ashbolt *et al.*, 2001). Consequently, the detection of  $\beta$ -galactosidase in isolate 6 by API 20E showed that this test cannot predict aerogenic ability.

One isolate (52) which resulted in typical colonies on L-EMB agar was compared with atypical L-EMB isolates, one which showed low dissimilarity (81-1, 0.08 level) and the other which was the most dissimilar (45, 0.44 level). This was done to determine the different discrepancies

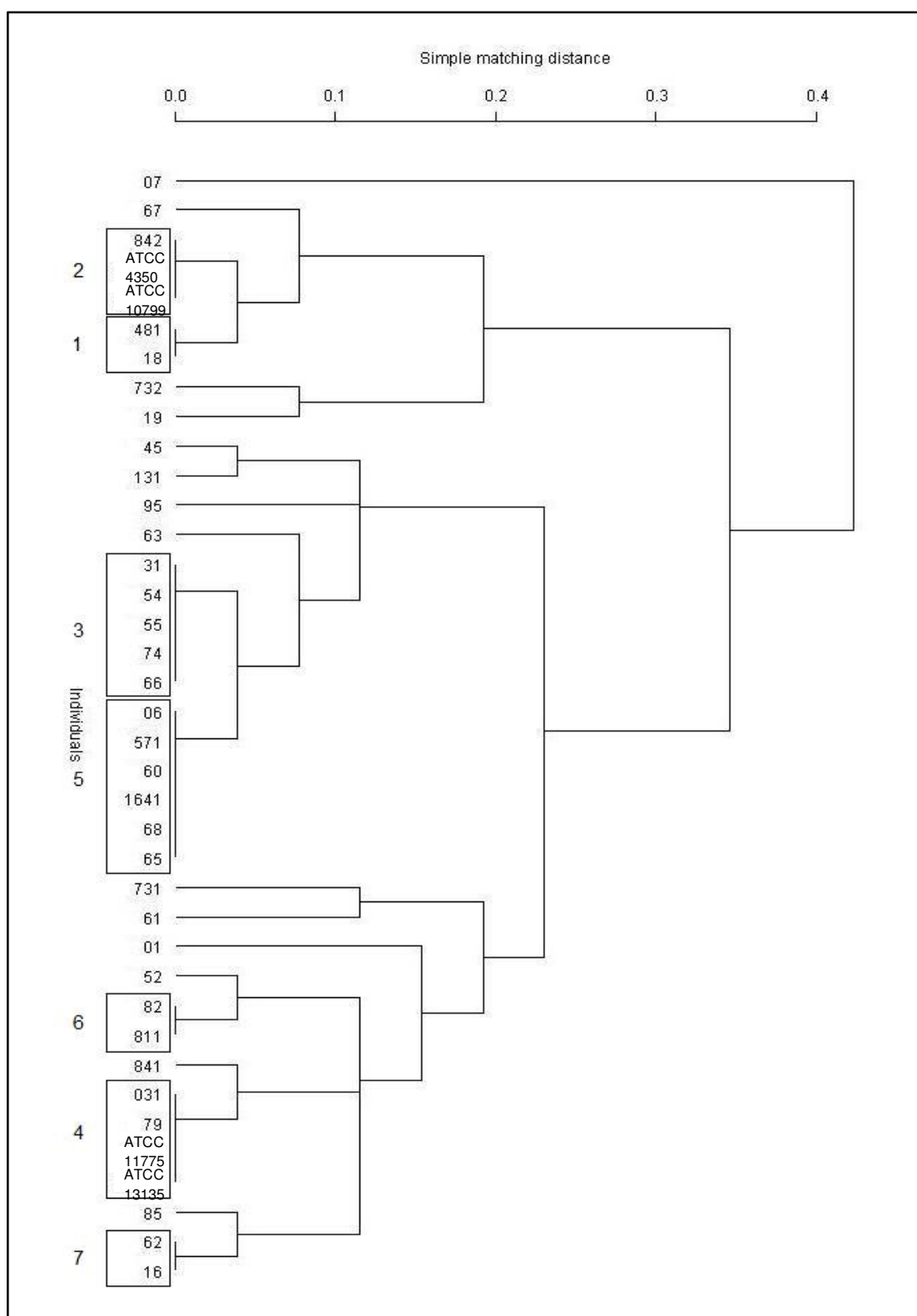
observed between the isolates from the atypical reactions and isolate 52, as well as to examine the possibility that the atypical nature would be reflected by the biochemical profile. The codes for the three organisms are given in Table 7.6.

**Table 7.6** API 20E codes for three *E. coli* isolates obtained from typical and atypical L-EMB reactions

Isolate		Code						
52	7	1	4	4	5	5	2	1
45	7	0	0	4	5	7	2	1
81-1	7	0	4	4	5	5	2	1

It is clear from Table 7.6 that isolate 81-1 was biochemically near identical to isolate 52 based on API data, even though these organisms produced different reactions on L-EMB agar. The only difference between the two organisms was the lack of ornithine decarboxylase in isolate 81-1. Since the metallic green colonies on L-EMB agar are associated with the fermentation of lactose, the absence of this enzyme in isolate 81-1 cannot account for the production of atypical colonies. Additionally, isolates 45, 52 and 81-1 all tested positive for  $\beta$ -galactosidase with API 20E. Therefore, their divergent reactions on L-EMB agar could not have been anticipated by the biochemical results generated by the API 20E test. Isolate 45, which exhibited a higher level of dissimilarity, differed from isolate 52 only in its inability to produce a positive indole reaction and its ability to ferment D-saccharose. It is possible that this organism utilises saccharose preferentially over its disaccharide sister molecule lactose, however, more empirical evidence would be necessary to prove such a theory. This could be determined through culturing the organism in a liquid medium containing both lactose and saccharose. The initial and final content of lactose and saccharose in the medium could be determined through formation of lactose and saccharose osazones during a glacial acetic acid/phenylhydrazine assay. Osazone crystals from different sugars can be discerned microscopically using a high-powered microscope.

The attempt to explain MTF behaviour by examining API 20E results can be further discounted by the observation that organisms isolated from typical reactions in EC broth with MUG and L-EMB also differed from one another, with some differing at the highest level (0.6 and 0.34, respectively) for this dendrogram. If the biochemical characteristics are so varied in isolates which elicited the same reactions in a given medium, the hope of finding meaningful differences between true positives and false negatives using these characteristics is to no avail. It is more likely that the differences observed here is attributable to the adaptations and variety observed in environmental isolates. This is supported by the fact that some of these *E. coli* isolates are so unique that API 20E could not securely or accurately identify them, and ribosomal protein data was necessary to acknowledge the identity of the isolates as *E. coli*. The Sokal and Michener simple matching dendrogram for *E. coli* isolates are shown as Fig. 7.4. Hypens were omitted but are used in text.



**Figure 7.4** Sokal and Michener simple matching dendrogram based on phenotypic characteristics of isolates identified as *E. coli* and four *E. coli* reference strains.

When comparing Fig. 7.3 and 7.4, it is immediately obvious that the same clusters occurred in the simple matching dendrogram, with some minor shifts in their positions, which are indicated here by the cluster numbers used in Fig. 7.3. Furthermore, the position of some of the isolates which were not associated with a cluster of maximally similar isolates shifted in the diagram. When the organisms are classified according to the broth from which they were isolated, as well as whether they resulted in typical or atypical reactions within this medium, it became immediately clear that typical and atypical isolates were yet again interspersed. The maximal dissimilarity level in this case was 0.44, singularly resulting from the segregation of isolate 7 away from the rest of the group. This isolate was identified by API 20E as *Klebsiella pneumoniae* spp. *pneumoniae* which accounts for this phenomenon, since the biochemical identification results were used to draw up the dendrogram. The organism was responsible for an anaerogenic reaction in EC broth with MUG. This isolate is compared with isolate 6, which produced the same reaction in EC broth with MUG and differs from isolate 7 on the level of maximal dissimilarity. It was found that the organisms differed due to the ability of isolate 7 to utilise citrate, its possession of urease and production of acetoin, and its ability to ferment inositol and amygdalin. In addition, isolate 6 possesses ornithine decarboxylase and produced a positive indole reaction. Therefore, these two organisms differ considerably despite their identical atypical reaction in EC broth with MUG. This reiterates once more the failure of API 20E results to predict the reactions of organisms in the MTF method.

The evidence presented here shows a compelling lack of associations between biochemical characteristics and exhibited reactions within various media of the MTF method. API 20E results are considered to be unrepresentative of the reactions from which isolates were observed. In the events where some level of speculation was warranted, more empirical evidence would need to be amassed to conclusively prove an association between a biochemical trait and the observed behaviour in the method.

#### Numerical dissimilarity based on ribosomal protein spectra of *E. coli* isolates

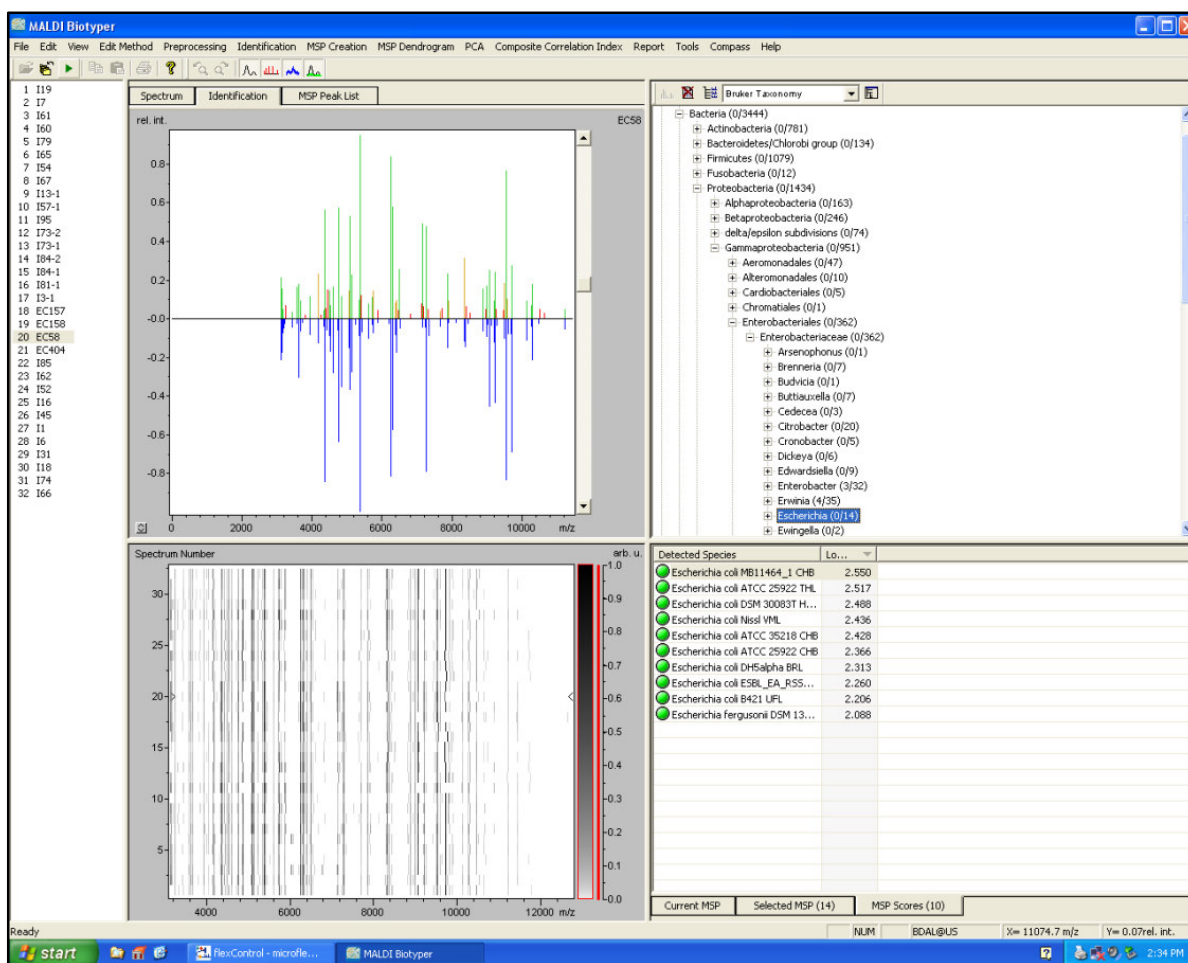
After MSPs were created for the 34 isolates of *E. coli*, as well as the four *E. coli* reference strains, their profiles were first compared with those of *E. coli* from the Bruker Taxonomy library. All isolates were matched to *E. coli*, in at least the best eight matches, with the majority (70.6%) of isolates (1, 3-1, 13-1, 18, 19, 31, 45, 48-1, 55, 57-1, 60, 64-1, 65, 66, 68, 73-1, 73-2, 74, 79, 81-1, 82, 84-2, 85 and 95) showing *Escherichia fergusonii* as one of the ten best matches in the ninth or tenth position. This is not surprising, since DNA-DNA hybridisation has demonstrated that *E. fergusonii* is the species genetically closest to *E. coli* (Maheux *et al.*, 2009). Additionally, since there were only eleven strains of *E. coli* within the Bruker Taxonomy library at the time of analysis, the likelihood of a better match with *E. fergusonii* exists due to a lack of suitable *E. coli* matches. This conclusion agrees with the report of Hsieh *et al.* (2008), who attributed low identification values to poor representation of certain organisms in the taxonomy library. Since the specificity of

the *tuf* primers have produced questionable results during this study, and have been reported by its designers to lack discriminatory ability between *E. coli*, *E. fergusonii* and *Shigella* (Maheux *et al.*, 2009), the results from the *uidA* PCR were deemed more suitable to confirm these organisms as *E. coli* and not *E. fergusonii*. Additionally, *uidA* has been described as 100% specific for the detection of *E. coli* by Maheux *et al.* (2009) and as more reliable marker of *E. coli* than  $\beta$ -D-glucuronidase expression by Heijnen & Medema (2006). Therefore, since all 34 isolates exhibited the presence of *uidA* in their genome, they were ruled as *E. coli* despite the discrepant *E. fergusonii* matches found in some cases. One isolate, 48-1, which exhibited a weak signal for *uidA* was identified as *E. fergusonii* in the tenth best match. This organism was, however, ruled as *E. coli* due to its high identification values of 2.317, 2.315 and 2.185 for the first, second and third best match, respectively, as well as its convincing identification as *E. coli* by API 20E (an identification of 99.5%).

These strains were not all isolated from the same step of the MTF method, and gave varying reactions within these steps: 4.2% was isolated from true positive L-EMB reactions, 8.3% from false negative EC broth with MUG reactions, 12.5% from false negative LST broth reactions, 16.7% from false negative L-EMB reactions, and 58.3% from true positive EC broth with MUG reactions. Therefore, this group represents aerogenic as well as anaerogenic strains, strains capable of reducing MUG, strains incapable of reducing MUG, strains growing as iridescent green colonies on L-EMB agar and, finally, strains which do not grow as iridescent green colonies on L-EMB agar. Therefore, it was concluded that these isolates would not have a detrimental effect on the dendrogram through skewing for a particular MTF reaction, and their spectra were included in the construction thereof.

The four reference strains were all highly matched with *E. coli*, but similarly showed *E. fergusonii* in lower likelihood matches. The Bruker Taxonomy matches with the spectra obtained for reference strain ATCC 11775 can be seen in Fig. 7.5. It can be seen in this figure that when the MSP for ATCC 11775 (EC58) was run against organisms within the Bruker Taxonomy library, the reference strain was matched with *E. coli* strains in the nine best matches. The tenth match was with *E. fergusonii*, at an identification value of 2.088. This finding vindicates the inclusion of strains identified in the ninth or tenth match as *E. fergusonii* into the dendrogram, as well as ruling these organisms *E. coli* despite their discrepant identifications.

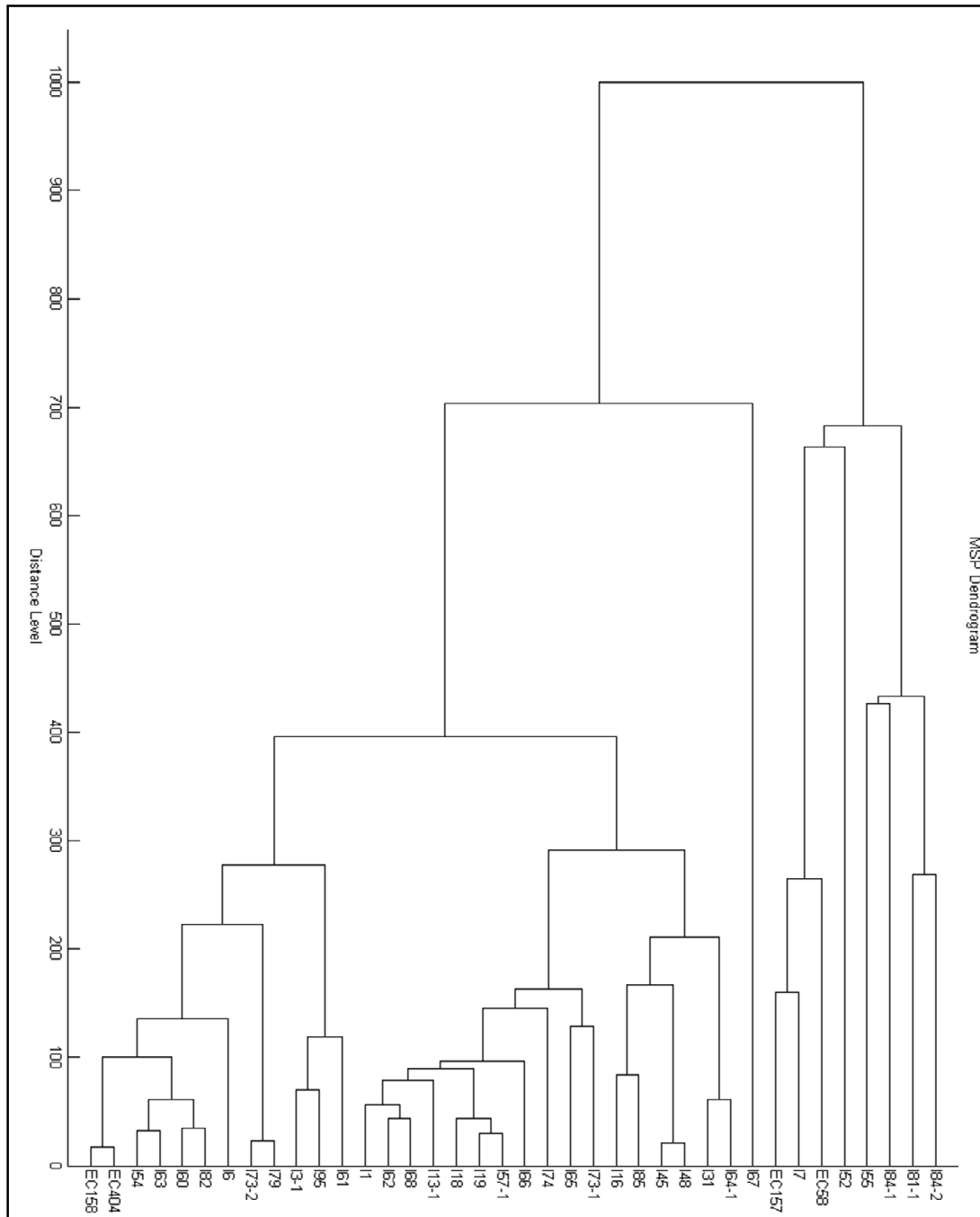
Other features seen in Fig. 7.5 include the comparison of the MSP spectrum for ATCC 11775 with its best match, *Escherichia coli* MB11464\_1 CHB, in the top left quadrant of the interface.



**Figure 7.5** Comparison of spectra from ATCC 11775 (EC58) MSP with organisms in the Bruker Taxonomy library

The top spectrum, that of ATCC 11775, shows multicoloured peaks: green peaks are peaks that agree fully with those of the best match (spectrum in blue), yellow peaks indicate peaks at the same mass but of different intensity to that of the best match and red peaks indicate peak shifts, where peaks do not occur at the same mass. The view in the bottom left quadrant of the interface is a gel-stack representation of the same similarities and differences between ATCC 11775 and its best match.

The dendrogram which was constructed based on the MSPs of the 34 *E. coli* isolates, as well as four reference strains of *E. coli*, is shown as Fig. 7.6. There are some features in this figure which are immediately visible. Firstly, the organisms occurred in two large clusters (isolate 84-2 to ATCC 4350 (EC157) and isolate 67 to ATCC 10799 (EC158) in the figure) which are maximally dissimilarity. The four reference strains did not occur together and clustered roughly in two categories: the first included ATCC 11775 (EC58) and ATCC 4350 (EC157), which were a distance level of 280 apart (28% dissimilar); while the second included ATCC 13135 (EC404) and ATCC 10799 (EC 158), which were at a distance level of 20 (2% dissimilar).



**Figure 7.6** MSP dendrogram of *E. coli* isolates and reference strains. *E. coli* isolate numbers are preceded by “I”.

These two clusters in Fig. 7.6 were at a maximal distance level from each other (100% dissimilar), and occurred in the two broad dissimilar clusters. Therefore, these positive control strains were interspersed between the *E. coli* isolates. The third observation that can be made from the dendrogram is that the organisms which had been identified as *E. fergusonii* were also interspersed with isolates which were identified as *E. coli* for all ten matches. Therefore, the



differences in spectral profiles of these “discrepant” isolates were not uniform, and strengthen the speculation that this misidentification was more likely due to a lack of good *E. coli* matches in the Bruker Taxonomy library. In addition, the cluster which is maximally dissimilar from the rest of the group comprises many of the isolates which constitute the top clusters (1 & 2) in the simple matching dendrogram for *E. coli* isolates (Fig. 7.4), which differs from the rest of the isolate clusters on a dissimilarity level of 0.35. These isolates are 7, 84-2 and one reference strain, ATCC 4350 (EC157). This may be purely by chance, since the other isolates which occur in the maximally dissimilar cluster in Fig. 7.6 are spread across the simple matching dendrogram. Since no similarities could be found between the Jaccard dissimilarity dendrogram for *E. coli* isolates (Fig. 7.3) and the dendrogram based on ribosomal protein spectra, this was assumed to be the case. This is not surprising, since the proteins which are measured by MALDI-TOF MS are burdened with the task of creating structural and heredity proteins and are most probably not directly responsible for the biochemical characteristics of an organism.

The *E. coli* isolates which were responsible for false negative reactions in LST broth are isolates 13-1, 48-1 and 95. Isolates 13-1 and 95 occurred in close proximity to each other, at a dissimilarity level of 80 (8%). Isolate 48-1 (denoted as 48 in Fig. 7.6), however, was dissimilar to these isolates at a level of 400 (40%). Since all these organisms elicited the same false negative reaction, namely growth but no gas production, this discrepancy cannot be explained by different reactions.

*E. coli* isolates which false negative results in EC broth with MUG are 6, 7, 18 and 19. These organisms did not cluster together, and although isolates 18 and 19 were only 6% dissimilar, the four organisms were divided between the two clusters differing maximally. Isolates 18 and 19, occurring in the “bottom” cluster (from 64-1 to EC158 in Fig. 7.6), were isolated from EC broth with MUG where they grew and produced gas, but could not produce fluorescence. The similarity in their reactions could possibly account for their low dissimilarity in Fig. 7.6, although such a conclusion at this point would be unsubstantiated. Isolate 7, which occurred in the “top” cluster (from 84-2 to EC157 in Fig. 7.6), was obtained from EC broth with MUG where growth and fluorescence was present in the absence of observable gas. Isolate 6, which occurred in the “bottom” cluster, exhibited an identical reaction. Since isolates 6 and 7 were shown to be maximally dissimilar based on ribosomal protein spectra, these proteins cannot be associated with the reactions produced in EC broth with MUG.

Organisms with true positive reactions in EC broth with MUG constitute the largest group, and is comprised of isolates 54, 55, 57-1, 60, 61, 62, 63, 64-1, 65, 66, 67, 68, 73-1, 73-2, 74, 79, 82, 84-1, 84-2 and 85. It can be observed in Fig. 7.6 that these organisms are uniformly interspersed throughout the dendrogram, and occur in both clusters. Therefore, isolates 55, 84-1 and 84-2 differ maximally from the rest of this group, despite all isolates exhibiting the same phenotypic reaction in EC broth with MUG.

True positive reactions on L-EMB were caused by two isolates of *E. coli*, namely isolates 31 and 52. Isolate 52 occurred in the top cluster, while isolate 31 occurred in the “bottom” cluster. Therefore, these two organisms were maximally dissimilar, which is in support of the earlier view that ribosomal proteins are not involved in the control of phenotypic behaviour within the steps of the MTF method and, as with API, cannot be used to describe or predict MTF reactions.

Finally, there were five isolates of *E. coli* which exhibited false negative reactions on L-EMB agar. These organisms are isolates 1, 3-1, 16, 45 and 81-1. Four of the isolates occurred in the “bottom” cluster of the dendrogram, while isolate 81-1 occurred in the “top” cluster. Therefore, this organism is maximally dissimilar to the others based on ribosomal protein spectra. This organism exhibited mucoid purple colonies on L-EMB agar, a reaction which similar to those found for isolate 45 (dark purple colonies) during the individual MTF analysis. A dissimilarity level of 30% was also found for isolate 16 when compared with isolate 1, despite the fact that these isolates both produced metallic green colonies when analysed individually (see section 8.4.1, Table 8.12). Isolate 3-1 was even more dissimilar to isolates 1, 16 and 45, at a level of 400 (40%).

Therefore, it can be concluded that the type of phenotypic reaction in the steps of the MTF method is not ribosomal protein-mediated. This is not to say that these proteins are not indirectly involved in the production of components which eventually do determine the phenotypic reactions. The conclusion simply means that the phenotypic expression of an organism, with a unique environmental history, within a medium which is complex in itself, could not be directly related here to its ribosomal proteins.

## 7.5. CONCLUSIONS

### 7.5.1. Numerical relatedness based on phenotypic characteristics

The most important conclusion which emerges from the results obtained from the API 20E analyses of the isolates is that the hydrolysis of 2-nitrophenyl- $\beta$ -D-galactopyranoside by organisms is not a good predictor of aerogenesis or anaerogenesis in MTF media. It is clear from the results presented that the mechanism of gas production from lactose in coliforms involves many factors, of which  $\beta$ -D-galactosidase is only one.

Biochemical results of the API 20E tests could not explain the differences between false positives and true negatives, or false negatives and true positives. This was reflected in the interspersed arrangement of isolates from typical and atypical reactions for a given medium in both Jaccard dissimilarity and Sokal and Michener simple matching dendrograms. Therefore, the characteristics determined by API 20E are inappropriate predictors of the reactions of organisms in media of the MTF method, and the use of these attributes to this end is not recommended.

An interesting phenomenon which requires further investigation is the observation that a large number of anaerogenic isolates could ferment more carbohydrates than their aerogenic

counterparts. It is possible that these organisms are able to utilise these carbohydrates preferentially over lactose, and consequently result in anaerogenic reactions in media of the MTF method.

### 7.5.2. Numerical relatedness based on ribosomal protein spectra

An important recommendation which emerged from the work done on *E. coli* MSPs was the necessity to have more strains of *E. coli* included in the Bruker Taxonomy database, since many of the *E. coli* isolates were identified as *E. fergusonii* in the lower match categories.

In addition, the spectrum of the ribosomal proteins of a given organism cannot be used as a predictor of the reactions the organism will elicit in MTF media. The *E. coli* isolates were arranged in an interspersed manner in the dendrogram, similar in pattern but not identical to the arrangements observed for API 20E results in the Jaccard and Sokal and Michener dendrograms. No similarities could be observed in the clustering of the *E. coli* isolates based on ribosomal protein spectra when they were compared with clusters observed in API 20E-based dendrograms, indicating that ribosomal proteins are not directly responsible for the biochemical profile of a given organism. Therefore, the use of ribosomal protein data in the prediction of (1) MTF reaction or (2) biochemical characteristics is strongly discouraged, since no relationship could be established here.

The failure by both API 20E and ribosomal protein spectra to predict reactions is not surprising, given the complexities involved in the reactions of the MTF. As mentioned before, every organism that was assayed has its own environmental history, occurs in a heterogeneous sample with many other organisms which result in their own reactions, and is assayed using media which is complex in itself.

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## CHAPTER 8

### CONTRIBUTION OF ORGANISMS ISOLATED FROM THE MULTIPLE TUBE FERMENTATION METHOD AND CLASSIFICATION OF METHODOLOGICAL RISK FOR UNDER- AND OVER-ESTIMATION OF COLIFORMS AND *E. COLI*

#### 8.1 ABSTRACT

A group of isolates (n=111) originally obtained from various steps of the multiple tube fermentation (MTF) method were separately analysed by MTF. This analysis was done to determine the individual contribution of each isolate to the observed reaction in each step of the method without the interference of co-occurring organisms or chemical substances in the environmental sample. These results, along with the phenotypic and molecular identifications of the isolates, were used to evaluate each isolate against a qualitative classification system. This system measured the maximum *methodological* risk for the under- or over-estimation of coliforms and *E. coli* posed by each isolate. The influence of 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG) and validation with Levine-eosin methylene blue (L-EMB) agar were also examined using the risk classification system. The results obtained for the individual analyses showed that several isolates did not reproduce the reactions they were initially isolated from, indicating that the “grab sample” effect of the isolation technique did not always isolate the organism responsible for the observed reaction. This is an important implication for the MTF method, where transference to consecutive steps of the method is based on the same principle. The qualitative methodological risk classification for the enumeration of coliforms showed that 50.5% of coliform isolates, and of these 88.2% of *E. coli* isolates, posed no risk for under-estimation of coliforms while 3.7% of isolates (none of them *E. coli*) occurred in the maximum risk category. Non-coliform isolates were not abundant; making up only 3.6% of isolates, but 75.0% did pose some degree of risk for the over-estimation of coliforms. The classification evaluating the methodological risk associated with the enumeration of *E. coli* showed that 50.0% of *E. coli* isolates posed no risk for under-estimation. The maximum risk category for under-estimation, relating to non-detection in the coliform enumeration steps, was assigned to 11.8% of *E. coli* isolates. The risk classification for *E. coli* over-estimation assigned 45.5% of non-*E. coli* isolates to the “no risk” category, while 11.7% of these isolates posed the maximum risk for over-estimation. It was determined that the absence of MUG from the assay would improve the overall accuracy of *E. coli* enumeration through a 20.6% increase in isolates with no risk for under-estimation, but a 1.3% decrease in isolates with no risk for *E. coli* over-estimation and 1.3% increase in isolates posing the maximum risk for over-estimation. The omission of L-EMB from the method did not show considerable changes in detection accuracy.

## 8.2 INTRODUCTION

Of the variety of methods used for the enumeration of coliforms and *Escherichia coli*, the MTF method remains the preferred enumeration method. This preference is in spite of the costly and labour intensive protocol of the method, as well as the long time before MTF results are available, which is offset by highly accurate results. This accuracy is primarily attributable to the selective hurdles incorporated within the different steps of the method.

Despite these selective hurdles, problems with the method have been reported in literature. False negative results frequently occur, which were attributed to the suppression of coliform aerogenicity by heterotrophic bacteria (Evans *et al.*, 1981; Edberg *et al.*, 1988). Evans *et al.* (1981) also reported inhibition of coliforms by selective ingredients in the media, while Szabo & Todd (1997) stated that the method does not have the ability to enumerate anaerogenic or late fermenting *E. coli* strains. It was further reported by Manafi (2000) that between 4% and 6% of *E. coli* strains cannot hydrolyse MUG, while 20% and 34% of  $\beta$ -D-glucuronidase negative strains were reported for environmental (Pisciotta *et al.*, 2002) and human clinical strains (Chang *et al.*, 1989), respectively. In addition, synergistic false positive reactions by coliforms have also been observed. Schiff *et al.* (1970) reported that *Proteus* and members of the enterococci group could react synergistically to create false positive reactions in lauryl sulphate tryptose (LST) broth. Furthermore, reports indicated that many other organisms resorting under genera within the coliform group possess the ability to hydrolyse MUG (see Table 2.7). The collective impact of these problems is a decrease in the accuracy of coliform and *E. coli* enumeration using the MTF. While false negative reactions will increase the degree of under-estimation, false positive reactions will increase over-estimation. However, a certain margin of error is realistic and expected for any microbiological method. Therefore, the propriety of a method is based on a relatively small risk of error rather than no error at all, and the judgment of the method is reliant on the size of this risk for error.

Additional to reports in literature, the results reported in Chapter 5 of this study also indicated the potential of MTF to fail in the detection of target organisms. In LST and brilliant green lactose bile (BGLB) broths, respectively 93.8% and 54.5% of coliform isolates would have remained undetected; while in *E. coli* (EC) broth with MUG 16.7% of *E. coli* isolates would not have been detected due to a lack of gas production or fluorogenic ability with MUG. On L-EMB agar, 71.4% of *E. coli* isolates would have been under-estimated through their failure to produce metallic green colonies. The potential of MTF to over-estimate *E. coli*, both through the detection of non-target organisms and synergistic false positives, was also observed. In EC broth with MUG and on L-EMB, respectively 25.9% and 33.3% of isolates from typical reactions were not *E. coli*. Some non-*E. coli* isolates with incomplete reactions in EC broth with MUG were also identified as potential sources of over-estimation if they occurred together, as their reactions could complement each other to result in a full false positive reaction. While it is accepted that these potential impacts

exist, they could not be conclusively proven in the previous work since these organisms were isolated from a heterogeneous population and not subjected to a full MTF analysis.

Given the constraints placed on the conclusions of the work in Chapter 5, the qualitative determination of the risk for under- and over-estimation of coliforms and *E. coli* is the focus of this study. Since the identities of 111 isolates obtained from the MTF method has already been established through both phenotypic and molecular analyses, they were considered ideal for determining the risk for under- and over-estimation. However, these organisms were isolated directly from the MTF media, and results reported in Chapter 7 showed that some organisms isolated from aerogenic reactions did not possess  $\beta$ -D-galactosidase. It was this observation which highlighted the possibility that the sampling technique which was employed to obtain the isolates may have isolated non-target organisms, since some  $\beta$ -D-galactosidase negative isolates were isolated from tubes where gas was produced.

The aim of the study was to use the individual contribution of isolates in MTF with a qualitative methodological risk classification system for the under- and over-estimation of coliforms and *E. coli* thereby determining the accuracy of the method with these isolates.

**Note:** during the reporting of these results, the terms “aerogenic” and “anaerogenic” have been used in the description of isolates. In this context, this does not refer to the oxygen requirements of the organism, but rather to the ability or inability to produce gas from lactose media.

## 8.3 MATERIALS AND METHODS

### 8.3.1 Determination of individual contribution of isolates to MTF reactions

The individual contribution of the 111 isolates from one of the four steps of the MTF method was investigated by subjecting each organism to the MTF protocol individually. These organisms were identified and characterised, both phenotypically and by molecular methods, as reported in Chapters 5 and 6.

All the isolates could not be analysed individually at the same time due to laboratory constraints. Therefore, isolates were grown overnight in 10 mL sterile nutrient broth (NB) (Biolab, Merck, Wadeville, South Africa) and incubated at 37°C. Subsequently, 500  $\mu$ L aliquots of this suspension were transferred in triplicate to cryogenic tubes (Greiner Bio-one, Neuburg, Germany) containing 500  $\mu$ L of sterilised 80% (v/v) glycerol, mixed and stored at -80°C.

After removal from cryogenic storage and thawing, 300  $\mu$ L was inoculated into 10 mL sterile NB and incubated at 37°C for 12 h. After mixing, the suspension was streaked out on nutrient agar (NA) (Biolab, Merck, Wadeville, South Africa) and *E. coli*/Coliform Chromogenic Agar (Oxoid, Basingstoke, Hampshire, UK) and incubated at 37°C for 24 h. Growth on chromogenic agar was



used to ensure the purity of the culture. A colony from the NA plate was transferred to 10 mL of sterile NB and incubated at 37°C for 12 h.

One loop of the overnight NB culture was transferred into 10 mL of sterile physiological saline solution (PSS) (0.85% m/v NaCl (Biolab)). The inoculated PSS served as “sample” to be tested by MTF. No further dilutions were made, since enumerative results were not the aim. The sample was inoculated in triplicate LST broth tubes, and incubated according to the guidelines of the MFHPB-19 MTF method (Christensen *et al.*, 2002). The reactions in LST broth were noted at 48 h. Reactions were evaluated for the presence or absence of gas and growth, indicated by turbidity in the medium. After 48 h, BGLB broth was inoculated with 48-h LST broth irrespective of the reaction observed in LST broth. The rationale was that organisms which do not elicit a positive reaction in LST broth may, due to co-occurring organisms which did produce a positive result, inadvertently be transferred to the next step, where they could possibly give false positive reactions. This methodology was followed to ascertain whether there is any step where an isolate can cause an incorrect reaction (observed in Chapter 5).

After the triplicate BGLB broth tubes were incubated at 37°C for 24 h, the individual reactions were observed and recorded. The reactions were judged for the presence or absence of gas and growth in the medium. EC broth with MUG was subsequently inoculated with the 24-h BGLB broth cultures and incubated at 44.5°C for 24 h. Once incubation was complete, the EC broth tubes were judged for the presence or absence of gas, growth and fluorescence (UV light at 365 nm). The reactions were recorded, and samples of all tubes streaked out onto L-EMB agar plates, which were subsequently incubated at 37°C for 24 h. Plates were examined for the colony appearance and this was recorded as final result.

Four reference strains of *E. coli*, ATCC 11775, ATCC 4350, ATCC 10799 and ATCC 13135, were subjected to the same procedure in Chapter 5 (see section 5.4.2). This was done to determine the individual behaviour of reference cultures of *E. coli*, and the results were used as positive controls for the experiment.

### **8.3.2 Classification of methodological risk for under- or over-estimation with MTF**

The results generated were used to determine the methodological risk which these isolates posed individually to the incorrect enumeration of coliforms, *E. coli*, or both with the MTF method.

The classification was divided into four risk profiles: (1) risk posed by isolates which cause under-estimation of coliforms; (2) risk posed by isolates which cause over-estimation of coliforms; (3) risk posed by isolates which cause under-estimation of *E. coli* and (4) risk posed by isolates which cause over-estimation of *E. coli*. A risk classification protocol was designed, as described below, for each profile. Categories were assigned based on the severity of the atypical reaction and subsequent under- or over-estimation.

### Risk classification for under- and over-estimation of coliforms

The individual reactions of the isolates in LST and BGLB broths causing under-estimation are listed in Table 8.1 in ascending order of severity, along with the risk category assigned to each reaction. All organisms assigned to a reaction in this table would be coliforms, since no under-estimation can be caused by anything other than coliforms with atypical reactions.

**Table 8.1** Reactions causing coliform “under-estimation” (U) and risk category assigned

Reaction in LST broth	Reaction in BGLB broth	Risk category
Gas and growth	Gas and growth	AU
Gas and growth	No gas but growth	BU
Gas and growth	No gas or growth	CU
No gas but growth	Gas and growth	DU
No gas but growth	No gas but growth	EU
No gas but growth	No gas or growth	FU
No gas no growth	-	GU

The reactions in Table 8.1 are listed in ascending order of severity. Therefore, risk category A represents no risk for under-estimation, the risk for under-estimation in category B is higher than that of category A, and the risk for under-estimation of coliforms is highest in category G. The reasoning for assigning lower risk to atypical reactions in BGLB than LST is simple: if coliforms produce gas and growth in LST broth, but not in BGLB broth, they are not enumerated as confirmed coliforms, but will still be enumerated as presumptive coliforms. If a coliform does not produce gas and growth in LST, however, it will be excluded from the coliform enumeration, both for presumptive and confirmed coliforms. There is a possibility, however, that an LST tube which is turbid with no gas could be misinterpreted as a positive reaction if the lack of gas is not observed by accident. Also, when this methodological risk classification was done, *E. coli* isolates were viewed as coliforms and were categorised as any other coliform.

The individual reactions of isolates in LST and BGLB which would lead to over-estimation of coliforms are shown in Table 8.2. The criteria are once again listed in ascending order of severity, and were each provided with a risk category. Organisms which cause over-estimation of coliforms must by definition be non-coliforms which are enumerated as coliforms.

**Table 8.2** Reactions causing coliform “over-estimation” (O) and risk category assigned

Reaction in LST broth	Reaction in BGLB broth	Risk category
No gas no growth		AO
No gas but growth	No gas or growth	BO
No gas but growth	No gas but growth	CO
No gas but growth	Gas and growth	DO
Gas and growth	No gas or growth	EO
Gas and growth	No gas but growth	FO
Gas and growth	Gas and growth	GO

The reactions in Table 8.2 are listed in order of increasing risk for over-estimation. There was a higher risk for over-estimation if non-coliforms could produce gas and growth in both LST and BGLB broths. They would then be enumerated as presumptive and confirmed coliforms. In the case when a non-coliform only produced gas and growth in LST broth, it will result only in the over-estimation of presumptive coliforms.

Risk classification for under- and over-estimation of *E. coli*

The individual reactions of *E. coli* isolates in the MTF method, which leads to under-estimation of *E. coli*, are given in Table 8.3. The reactions are listed in order of increasing risk for under-estimation. The under-estimation of *E. coli* can only arise from *E. coli* isolates with anomalous reactions in this media.

**Table 8.3** Reactions causing *E. coli* “under-estimation” (u) and risk category assigned

Reaction in EC broth with MUG	Reaction on L-EMB agar	Risk category
Gas, growth and fluorescence	Metallic green colonies	au
No gas but growth and fluorescence	Metallic green colonies	bu
Gas and growth but no fluorescence	Metallic green colonies	cu
No gas or fluorescence but growth	Metallic green colonies	du
No gas, growth or fluorescence	Metallic green colonies	eu
Gas, growth and fluorescence	Not metallic green colonies	fu
No gas but growth and fluorescence	Not metallic green colonies	gu
Gas and growth but no fluorescence	Not metallic green colonies	hu
No gas or fluorescence but growth	Not metallic green colonies	iu
No gas, growth or fluorescence	Not metallic green colonies	ju
Reaction in LST and BGLB broth	Reaction on L-EMB agar	Risk category
Non-detection in LST and/or BGLB <sup>‡</sup>	n/a	ku

<sup>‡</sup>Non-detection refers to the absence of growth and/or gas in one or both media

The reactions listed in Table 8.3 which result in the production of metallic green colonies on L-EMB agar in the final step pose a lower risk for under-estimation than those which end in “not metallic green” (NMG) colonies on L-EMB agar. This is because L-EMB agar is the confirmatory step at the end of the method, and its purpose is to determine whether the *E. coli* enumeration from EC broth with MUG was correct. If metallic green colonies are found, the enumeration is considered correct. If NMG colonies are found, the isolate is not considered to be *E. coli* and the enumeration from EC broth with MUG is amended. For this reason a completely normal reaction in EC broth with MUG (gas, growth and fluorescence) but with atypical colonies on L-EMB is considered a higher risk (category f) than an anomalous EC broth with MUG reaction which produces metallic green colonies on L-EMB agar. Since results from L-EMB agar can be used to overrule those obtained by EC broth with MUG, categories e and j were included. Both categories describe no reaction in EC broth with MUG, and either metallic green (category e) or NMG (category j) colonies on L-EMB.

The absence of gas with growth and fluorescence in EC broth with MUG was considered a lower risk for under-estimation than the absence of fluorescence with the presence of gas and growth. This is due to the trouble in visually observing the lack gas production in a tube which is turbid with organism growth. The absence of fluorescence, although some judgments in this respect are very difficult, is mostly easier to observe due to the absence of the bright blue fluorescent reaction. Therefore, it was accepted for this risk classification that the likelihood of transference from a tube with no gas production is higher than that of a tube with no fluorescent reaction.

The final category in Table 8.3, category k, represents the most serious methodological risk classification for the under-estimation of *E. coli*. If this organism is not detected in LST and BGLB broth it will not only be under-estimated as a coliform, but also as *E. coli*. Technically, this category should also be subdivided into categories of severity, which should, in turn, correspond to each EC broth with MUG and L-EMB permutation. However, the analysis becomes extremely intricate if this is the case. It was therefore decided that any failure by LST or BGLB broth would result in non-transference to EC broth with MUG, which was assigned the highest possible risk for under-estimation of *E. coli*.

The omission of MUG from EC broth was evaluated by excluding fluorescence from the risk categorisation. This was done to determine the influence of MUG on the under-estimation of *E. coli*. The risk categorisation for this evaluation is presented in Table 8.4. Different risk categories were assigned in order to draw meaningful comparisons with the results obtained from the categorisation done according to Table 8.3.

**Table 8.4** Risk categorisation to evaluate the influence of MUG (M) on *E. coli* “under-estimation”

Reaction in EC broth “without MUG”	Reaction on L-EMB agar	Risk category
Gas and growth	Metallic green colonies	Mi
No gas but growth	Metallic green colonies	Mii
No gas or growth	Metallic green colonies	Miii
Gas and growth	Not metallic green colonies	Miv
No gas but growth	Not metallic green colonies	Mv
No gas or growth	Not metallic green colonies	Mvi
Reaction in LST and BGLB broth	Reaction on L-EMB agar	Risk category
Non-detection in LST and/or BGLB <sup>‡</sup>	n/a	Mvii

<sup>‡</sup>Non-detection refers to the absence of growth and/or gas in one or both media

The omission of the L-EMB agar step, as confirmation of the enumeration by EC broth with MUG, was also evaluated by excluding the type of colonies obtained on L-EMB agar from the risk categorisation. This was done to determine what influence the exclusion of L-EMB would have on the under-estimation of *E. coli*. The risk categorisation done with the exclusion of L-EMB agar results are presented in Table 8.5.

**Table 8.5** Risk categorisation to evaluate the influence of L-EMB (L) on *E. coli* “under-estimation”

Reaction in EC broth with MUG	Risk category
Gas, growth and fluorescence	Li
No gas but growth and fluorescence	Lii
Gas and growth but no fluorescence	Liii
No gas or fluorescence but growth	Liv
No gas, growth or fluorescence	Lv
Reaction in LST and BGLB broth	Risk category
Non-detection in LST and/or BGLB <sup>‡</sup>	Lvi

<sup>‡</sup>Non-detection refers to the absence of growth and/or gas in one or both media

The individual reactions of non-*E. coli* isolates in EC broth with MUG and L-EMB agar, which causes the under-estimation of *E. coli*, are given in Table 8.6. The reactions are listed in order of increasing risk severity. The over-estimation of *E. coli* can only occur when organisms not belonging to *E. coli* cause typical reactions which result in their enumeration as *E. coli*.

**Table 8.6** Reactions causing *E. coli* “over-estimation” (o) and risk category assigned

Reaction in LST and BGLB broth	Reaction on L-EMB agar	Risk category
Non-detection in LST and/or BGLB <sup>‡</sup>	n/a	ao
Reaction in EC broth with MUG	Reaction on L-EMB agar	Risk category
No gas, growth or fluorescence	None/Not metallic green colonies	bo
No gas or fluorescence but growth	Not metallic green colonies	co
Gas and growth but no fluorescence	Not metallic green colonies	do
No gas but growth and fluorescence	Not metallic green colonies	eo
Gas, growth and fluorescence	Not metallic green colonies	fo
No gas, growth or fluorescence	Metallic green colonies	go
No gas or fluorescence but growth	Metallic green colonies	ho
Gas and growth but no fluorescence	Metallic green colonies	io
No gas but growth and fluorescence	Metallic green colonies	jo
Gas, growth and fluorescence	Metallic green colonies	ko

<sup>‡</sup>Non-detection refers to the absence of growth and/or gas in one or both media

The five reactions listed in Table 8.6 which result in metallic green colonies on L-EMB agar are considered to pose a higher risk for over-estimation than those which resulted in NMG colonies on L-EMB. This classification was done since results from L-EMB agar can overrule the enumeration with EC broth with MUG. Therefore, non-*E. coli* isolates which resulted in metallic green colonies on L-EMB agar would, irrespective of their reaction in EC broth with MUG, be enumerated as *E. coli*. For this reason, category k was included, although these reactions were not expected.

In the case of *E. coli* over-estimation, the absence of fluorescence was considered a lower risk for over-estimation than the lack of gas production, as the lack of fluorescence is easily observed. The lack of gas production can be concealed by turbidity in the medium, and an incorrect assumption of the presence gas production can easily occur.

As was done for the under-estimation of *E. coli*, the omission of MUG from EC broth was evaluated by excluding fluorescence from the risk categorisation for over-estimation of *E. coli*. The risk categorisation for this evaluation is presented in Table 8.7. Different risk categories were assigned in order to draw meaningful comparisons with the results obtained from the analysis done according to Table 8.6.

Additionally, the omission of the L-EMB agar step, as confirmation of the enumeration by EC broth with MUG, was also evaluated for the over-estimation of *E. coli*. This was done by excluding the type of colonies obtained on L-EMB agar from the risk categorisation. The risk categorisation done with the exclusion of L-EMB agar results is presented in Table 8.8.

**Table 8.7** Risk categorisation to evaluate the influence of MUG (M) on *E. coli* “over-estimation”

Reaction in LST and BGLB broth	Reaction on L-EMB agar	Risk category
Non-detection in LST and/or BGLB <sup>‡</sup>	n/a	MI
Reaction in EC broth “without MUG”	Reaction on L-EMB agar	Risk category
No gas or growth	None/Not metallic green colonies	MII
No gas but growth	Not metallic green colonies	MIII
Gas and growth	Not metallic green colonies	MIV
No gas or growth	Metallic green colonies	MV
No gas but growth	Metallic green colonies	MVI
Gas and growth	Metallic green colonies	MVII

<sup>‡</sup>Non-detection refers to the absence of growth and/or gas in one or both media

**Table 8.8** Risk categorisation to evaluate the influence of L-EMB (L) on *E. coli* “over-estimation”

Reaction in LST and BGLB broth	Risk category
Non-detection in LST and/or BGLB <sup>‡</sup>	LI
Reaction in EC broth with MUG	Risk category
No gas, growth or fluorescence	LII
No gas or fluorescence but growth	LIII
Gas and growth but no fluorescence	LIV
No gas but growth and fluorescence	LV
Gas, growth and fluorescence	LVI

<sup>‡</sup>Non-detection refers to the absence of growth and/or gas in one or both media

The number of isolates in each category obtained for each of the risk profiles was calculated, to ascertain the amount of organisms causing incorrect enumeration and, more importantly, the severity of incorrect enumeration for each risk profile.

## 8.4 RESULTS AND DISCUSSION

### 8.4.1 Determination of individual contribution of pure isolates to reactions in MTF media

The individual contribution of organisms which were originally isolated from LST broth to the reactions in MTF media is given in Table 8.9. The identity of each isolate as MALDI-TOF MS is also shown. The individual contributions of isolates originally from BGLB broth follow directly (Table 8.10). If the three triplicates of any reaction did not agree, the two which corresponded were used to rule out the third.



**Table 8.9** Individual reactions in MTF media elicited by isolates from LST broth<sup>‡</sup>

Isolate	Identity	Reaction in LST			Reaction in BGLB			Reaction in EC with MUG			Reaction on L-EMB		
Original atypical reactions in LST broth: growth but no gas													
4	<i>A. hydrophila</i>	GR	GR	GR	GR	GR	GR	-	-	-	-	-	-
5	<i>Entb. aerogenes</i>	GR	GR, G	GR, (G)	GR, G	GR, G	GR, G	(GR), (G)	(GR), (G)	-	-	-	-
11-1	<i>Cit. braakii</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG
11-2	<i>M. morganii</i>	GR, G	GR	GR	GR, G	GR	GR	GR, G, F	GR, G, F	-	MG	MG	-
12	<i>Cit. braakii</i>	GR, (G)	GR, (G)	GR, G	GR, G	GR, (G)	GR, G	-	-	-	MG	MG	MG
13-1	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG
13-2	<i>Prov. alcalifaciens</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG
14	<i>Prov. alcalifaciens</i>	(GR)	(GR)	(GR)	(GR)	(GR)	GR	-	-	-	-	-	-
15	<i>K. pneumoniae</i>	(GR)	(GR)	(GR)	(GR)	(GR)	(GR)	-	-	-	NMG	NMG	NMG
17	<i>Entb. asburiae</i>	GR	GR	GR	GR, G	GR, G	GR, G	-	-	-	MG	MG	MG
21	<i>M. luteus</i>	(GR)	(GR)	(GR)	(GR)	(GR)	(GR)	-	GR, G	-	-	NMG	-
26	<i>B. pseudofirmus</i>	GR, G	GR, G	GR, G	-	-	-	-	-	-	-	-	-
27	<i>K. pneumoniae</i>	GR	GR	GR, G	GR	GR	GR, (G)	-	GR	GR, G	-	NMG	NMG
28	<i>K. pneumoniae</i>	GR	GR	GR	-	-	-	-	-	-	-	-	-
30	<i>S. marcescens</i>	GR, (G)	GR, (G)	GR, (G)	GR	GR	GR	-	-	-	-	-	-
35	<i>S. marcescens</i>	GR	GR	GR	-	-	GR	-	-	-	-	-	-
39	<i>H. alvei</i>	GR, (G)	GR, (G)	GR, (G)	GR, G	GR, G	GR, G	-	-	-	-	-	-
41	<i>Entb. cloacae</i>	GR, (G)	GR, (G)	GR, (G)	GR, G	GR, G	GR, G	GR, G	GR	GR, G	NMG	NMG	NMG
42	<i>Entb. asburiae</i>	GR, (G)	GR, (G)	GR, (G)	GR, (G)	GR, (G)	GR, (G)	GR	GR, G	GR, G	NMG	NMG	NMG
43	<i>Entb. asburiae</i>	GR, (G)	GR, (G)	GR, (G)	GR, (G)	GR, (G)	GR, (G)	GR	GR, G	GR	NMG	NMG	NMG
44	<i>Prov. rettgeri</i>	GR	GR	GR	GR	GR	GR	-	-	-	-	-	-
46	<i>K. pneumoniae</i>	GR, (G)	GR, (G)	GR, (G)	GR, G	GR, G	GR, G	GR, F	GR, F	GR, G, F	NMG	NMG	NMG
47	<i>Cit. braakii</i>	GR, (G)	GR, (G)	GR, (G)	GR, G	GR, G	GR, G	GR, F	GR	GR	NMG	NMG	NMG

**Table 8.9 continued**

Table 8.9 continued

Isolate	Identity	Reaction in LST			Reaction in BGLB			Reaction in EC with MUG			Reaction on L-EMB		
Original atypical reactions in LST broth: growth but no gas													
48-1	<i>E. coli</i>	GR, G	GR, G	GR,G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	MG	MG	MG
48-2	<i>S. marcescens</i>	GR, G	GR, G	GR, G	GR	GR	GR	GR, G, F	GR, G, F	-	NMG	NMG	NMG
50	<i>Entb. asburiae</i>	GR, (G)	GR, (G)	GR, (G)	GR, G	GR, G	GR, G	GR	GR	GR	NMG	NMG	NMG
53	<i>K. pneumoniae</i>	GR, G	GR, G	GR, G	GR, (G)	GR, (G)	GR, (G)	GR, G, F	GR, G, F	GR,(G),F	MG	MG	MG
56	<i>Entb. cloacae</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	-	-	MG	-	-
59	<i>K. pneumoniae</i>	GR, G	GR, G	GR, G	GR	GR	GR	-	-	-	-	-	-
80	<i>Cit. freundii</i>	GR	GR, G	GR	GR	GR, G	GR, G	-	GR, G, F	GR, G, F	-	MG	MG
86	<i>R. ornithinolytica</i>	GR	GR	GR	(GR)	(GR)	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG
87	<i>Entb. kobei</i>	GR	GR	GR, G	GR, G	GR, G	GR, G	-	-	-	-	-	-
95	<i>E. coli</i>	GR	GR	GR	GR, (G)	GR, (G)	GR, (G)	-	GR, F	GR, F	-	MG	MG
Original typical reactions in LST broth: growth and gas													
101	<i>Prot. vulgaris</i>	GR, (G)	GR, (G)	GR, (G)	GR, (G)	GR, (G)	GR, (G)	-	-	-	-	-	-
109	<i>Prov. stuartii</i>	GR	GR	GR	GR	GR	GR, (G)	GR	GR	GR	NMG	NMG	NMG

\*Reactions in brackets indicate an unacceptably weak reaction

GR: growth (turbidity), G: gas production, F: fluorescence, MG: metallic green colonies, NMG: "not metallic green" colonies, - : no reaction detected

**Table 8.10** Individual reactions in MTF media elicited by isolates from BGLB broth<sup>‡</sup>

Isolate	Identity	Reaction in LST			Reaction in BGLB			Reaction in EC with MUG			Reaction on L-EMB		
Original atypical reactions in BGLB broth: growth but no gas													
22	<i>M. morganii</i>	GR, (G)	GR, (G)	GR, (G)	GR, (G)	GR, (G)	GR, (G)	-	GR, G	-	-	NMG	-
23	<i>M. morganii</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR	GR, G, F	GR, G, F	NMG	MG	MG
24-1	<i>Entb. asburiae</i>	GR	GR	GR, (G)	GR, G	GR, G	GR, G	(GR), (G)	(GR)	(GR)	-	-	-
24-2	<i>M. morganii</i>	GR, (G)	GR	GR	GR	GR	GR, (G)	(GR)	(GR)	(GR)	-	-	-
25	<i>Entb. radicincitans</i>	GR	GR	GR	GR	GR	GR	-	-	-	-	-	-
29	<i>R. ornithinolytica</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	NMG	NMG	NMG
34	<i>Entb. asburiae</i>	GR, (G)	GR, (G)	GR, (G)	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G	MG	MG	NMG
38	<i>K. oxytoca</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	NMG	NMG	NMG
40	<i>K. oxytoca</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	NMG	NMG	NMG
51	<i>R. ornithinolytica</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, F	GR, F	GR, F	MG	MG	MG
75	<i>R. ornithinolytica</i>	GR, G	GR	GR	GR, G	GR	GR	GR, G, F	-	-	MG	-	-
92	<i>Cit. braakii</i>	GR	GR	GR	-	-	-	-	-	-	-	-	-
Original typical reactions in BGLB broth: growth and gas													
98	<i>Prov. stuartii</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR	GR	GR	NMG	NMG	NMG
99	<i>Prot. vulgaris</i>	GR	GR	GR	(GR), (G)	(GR), (G)	(GR), (G)	-	-	-	-	NMG	NMG
100	<i>Prot. mirabilis</i>	GR, (G)	GR	GR	GR, (G)	GR, (G)	GR, (G)	GR	GR	GR	NMG	NMG	NMG
102	<i>Prot. vulgaris</i>	GR	GR	GR	GR, (G)	GR, (G)	GR, (G)	-	-	-	-	-	-
103	<i>Prot. mirabilis</i>	GR	GR, (G)	GR	GR, (G)	GR, (G)	GR, (G)	GR	GR	GR	NMG	NMG	NMG
104	<i>Prov. stuartii</i>	(GR)	(GR)	(GR)	-	-	-	GR	GR	GR	NMG	NMG	NMG
105	<i>Prot. mirabilis</i>	(GR)	(GR)	(GR)	GR, (G)	GR, (G)	GR, (G)	GR	GR	GR	NMG	NMG	NMG
106	<i>Prot. mirabilis</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR	GR	GR	NMG	NMG	NMG
107	<i>Prot. vulgaris</i>	GR	GR, (G)	GR, (G)	GR, (G)	GR, (G)	GR, (G)	-	-	-	-	NMG	-
108	<i>K. pneumoniae</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR	GR	GR	NMG	NMG	NMG

<sup>‡</sup>Reactions in brackets indicate an unacceptably weak reaction

GR: growth (turbidity), G: gas production, F: fluorescence, MG: metallic green colonies, NMG: "not metallic green" colonies, - : no reaction detected

### Isolates originally producing typical and atypical reactions in LST broth

The results in Tables 8.9 and 8.10 show that the individual reactions of the isolates did not necessarily correspond with their reactions in the medium from which they were originally isolated.

Of the atypical organisms isolated from LST, 19 (57.6%) exhibited both growth and some degree of gas production when they were inoculated individually into LST broth. The probable higher starting inoculum concentrations used in the individual MTF analyses, when compared to initial river water MTF analyses, as well as the absence of any competing organisms, may have contributed to the discrepant reactions observed during some individual MTF analyses. The majority (94.7%) of these isolates are coliforms and consequently exhibited the expected reaction in LST. Only one, *Bacillus pseudofirmus* (isolate 26), was not a coliform. Interestingly, a coliform isolate from a typical LST broth reaction, *Providencia stuartii* (isolate 109), failed to produce gas when inoculated individually into LST broth. The reason for this is unclear, but it is possible that this coliform is an anaerogenic strain which was originally in co-habitation with one or more aerogenic organisms. This observation is an important indication of the “grab-sample” effect which is inevitably associated with the sampling technique employed to isolate these organisms. Therefore, the initial reaction from which organisms were isolated is not necessarily the reaction elicited by the individual organism.

Based on the results in Table 8.9, 18 originally atypical coliforms were reassigned as organisms exhibiting typical reactions in LST broth. Additionally, the originally typical isolate 109 was reassigned as atypical. These changes resulted in the number of coliform isolates from LST broth which produced typical reactions in LST broth increasing from two (6.1%) to 20 (60.6%). In contrast, the number of coliform isolates which could not produce typical reactions in LST decreased from 31 (93.9%) to 13 (39.4%). This proportion of anaerogenic coliforms does not agree with the findings by LeChevallier *et al.* (1983), who found that anaerogenic isolates from lauryl tryptose broth (LTB) was 91.5% comprised of organisms from the coliform genera. However, it should be remembered that the inoculation of individual isolates into a medium removes any factors present in the environmental sample which could influence the reaction of the organism. Furthermore, the observed reduction in anaerogenic coliforms when isolates were inoculated individually into LST broth supports the reports of workers who cite the inhibition by indigenous heterotrophic bacteria present in samples as one of the reasons for the failure of coliforms to produce gas (Evans *et al.*, 1981; Edberg & Edberg, 1988). It is also possible that these organisms were injured when they were originally isolated, which increased their lag time (Niemela *et al.*, 2003), but subsequently recovered during the various subculturing steps.

Two of the three *E. coli* organisms (13-1 and 48-1) isolated from atypical LST broth reactions produced both growth and gas when they were individually inoculated into this medium. In addition, these organisms exhibited both growth and gas production in BGLB broth. Since these

organisms were originally isolated from LST broth where no gas was produced, it must be concluded that these organisms were either sublethally injured during their first inoculation, or suppressed by heterotrophic bacteria in the sample. Isolate 13-1 was isolated in co-occurrence with *Providencia alcalifaciens* (isolate 13-2), which was also able to produce gas in isolation. It is, therefore, likely that the suppression of isolates 13-1 and 13-2 was due to injury, or inhibition by another organism. It is also possible that these organisms mutually suppressed each other's ability to ferment lactose. These *E. coli* isolates also produced metallic green colonies on L-EMB agar, however, while isolate 13-1 exhibited growth, gas and fluorescence in EC broth with MUG, isolate 48-1 could only grow and produce gas. This isolate would only be enumerated if the L-EMB agar step was used to amend *E. coli* enumeration done by EC broth with MUG. The assumption that results from L-EMB can overrule these enumerations should be used cautiously, however: this is only the case if all EC broth with MUG tubes, typical and atypical, are streaked out on L-EMB agar; a practice which is not commonplace in most laboratories and is not recommended by the official method (Christensen *et al.*, 2002).

The remaining *E. coli* isolate (95) grew, but failed to produce gas in LST broth. Additionally, the isolate grew but produced unacceptably low amounts of gas in BGLB broth. This isolate is presumably an anaerogenic strain, since it was also originally isolated from LST broth which exhibited no gas production. The production of a small amount of gas in BGLB broth, however, indicates that the organism could possess a limited ability to ferment lactose. The likelihood of this production of gas in BGLB broth being due to recovery in LST broth is small, since the organism had been subjected to several subculturing steps. However, the lack of some component in the hydrogenlyase reaction could possibly be responsible for this phenomenon. The work done by Gest and Peck (1955) suggested that some organisms may have defective formic dehydrogenase systems. These workers used this concept to explain their observation that some organisms which possessed the hydrogenase enzyme showed high hydrogenlyase activity when combined with an organism with functional formic dehydrogenase while others could produce only half of that amount of hydrogenlyase. They further postulated that the defect is most probably the absence of an electron carrier, which links formic dehydrogenase and hydrogenase to facilitate the production of gas from formic acid, an intermediate in the hydrolysis of carbohydrates such as glucose or lactose (Ordal & Halvorson, 1939). The lack of gas production by isolate 95 was once again observed in EC broth with MUG, where the organism grew and produced fluorescence, but failed to produce gas. This is further evidence refuting the theory that its anaerogenesis, observed in LST broth, was due to injury or suppression. The organism did, however, produce metallic green colonies on L-EMB agar, which would have overruled the incorrect enumeration made as a consequence of the incorrect reaction in EC broth with MUG.

Seven coliforms isolated from LST broth (11-1, 11-2, 13-2, 46, 48-2, 53 and 80) exhibited growth, gas and fluorescence in EC broth with MUG and, furthermore, five of these organisms (11-1, 11-2, 13-2, 53 and 80) produced metallic green colonies on L-EMB agar. These isolates are

very important, since their presence would result in an over-estimation of *E. coli* even with the confirmatory step on L-EMB agar. It is encouraging that isolate 11-2 failed to produce gas in LST and BGLB broths, and isolate 80 failed to produce gas in LST broth. This would have excluded these isolates from EC broth with MUG in a “normal” MTF assay. Isolate 48-2 also did not produce gas in BGLB broth, but since it did not result in metallic green colonies on L-EMB agar, its accidental transference to EC with MUG would not be as detrimental to enumeration accuracy.

#### Isolates originally producing typical and atypical reactions in BGLB broth

The results of isolates from BGLB broth, presented in Table 8.10, shows a similar increase in aerogenic reactions by organisms initially isolated from BGLB tubes with no gas production. Of the 12 originally atypical isolates, which were all coliforms, eight (66.7%) exhibited growth and some degree of gas production in BGLB broth. This observation is further support for the theory that either coliform suppression by other indigenous bacteria, or sub-lethal injury, is responsible for anaerogenesis in some coliforms. Seven of these isolates were also able to produce growth and gas in LST broth. Interestingly, isolate 24-1 could not produce gas in LST broth. Since this organism exhibited strong gas production in BGLB broth in the individual analysis, this observation is not due to anaerogenesis. If this isolate was inadvertently transferred to BGLB, perhaps in co-habitation with an aerogenic coliform, it would correctly be enumerated as a confirmed coliform.

One of the isolates originally isolated from a typical BGLB broth reaction, isolate 104 (*Providencia stuartii*), failed to produce gas or growth in BGLB. Since this organism produced reactions in subsequent steps of the MTF, the possibility that the isolate died was ruled out. It is possible that this organism co-occurred with an aerogenic organism when it was first isolated, and once again highlights the unpredictability of the isolation technique. Isolate 109, isolated from a typical LST broth reaction, also failed to produce gas in both LST and BGLB broths when it was inoculated individually. This observation is of interest here because this organism was also identified as *P. stuartii*, which could indicate that this organism has a tendency for anaerogenesis. This is also in agreement with the observation in Chapter 7 that *Providencia* isolates did not hydrolyse 2-nitrophenyl- $\beta$ -D-galactopyranoside and the subsequent theory that these organisms do not readily produce gas but are extremely proficient at growing during isolation subculturing. However, this is presumably not a defect ubiquitous to the species: isolate 98, also *P. stuartii*, produced growth and gas in both its initial and individual reaction in BGLB broth.

If the reactions of these eight previously atypical organisms, and one previously typical organism, were reassigned based on the results in Table 8.10, the number of coliforms isolated from BGLB which exhibit typical reactions would increase from ten (45.5%) to 17 (77.3%).

Two of the organisms, isolates 23 and 34, identified respectively as *Morganella morganii* and *Enterobacter asburiae*, were able to exhibit growth, gas production and fluorescence in EC broth with MUG. Furthermore, these isolates produced metallic green colonies on L-EMB agar.

These organisms would result in over-estimation of *E. coli*, since they also caused growth and gas in LST and BGLB. These are examples of perfect false positive *E. coli* organisms in MTF.

The results of individual contributions by isolates from EC broth with MUG are shown in Table 8.11, along with their identification as determined by MALDI-TOF MS.



**Table 8.11** Individual reactions in MTF media elicited by isolates from EC broth with MUG<sup>‡</sup>

Isolate	Identity	Reaction in LST			Reaction in BGLB			Reaction in EC with MUG			Reaction on L-EMB		
Original atypical reactions in EC broth with MUG: growth and fluorescence, but no gas													
6	<i>E. coli</i>	GR	GR	GR	GR	GR	GR	GR, F	GR, (G),F	GR, F	MG	NMG	MG
7	<i>E. coli</i>	GR	GR	GR	GR	GR	GR	GR,(G),F	GR, F	GR, F	MG	MG	MG
69	<i>Entb. asburiae</i>	GR, G	GR, G	GR, G	GR, (G)	GR, G	GR, (G)	-	GR, G	-	-	MG	-
70	<i>Entb. asburiae</i>	GR, G	GR, G	GR, G	GR, (G)	GR, (G)	GR, (G)	-	-	-	-	-	-
Original atypical reactions in EC broth with MUG: growth, no gas and no fluorescence													
8	<i>K. pneumoniae</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR	GR	GR	NMG	NMG	NMG
37	<i>B. pseudofirmus</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	NMG	NMG	NMG
72	<i>Entb. kobei</i>	GR, (G)	GR, (G)	GR, (G)	GR, (G)	GR, G	GR, (G)	-	-	-	-	-	-
94	<i>Cit. freundii</i>	GR, G	GR, G	GR, G	GR, (G)	GR, (G)	GR, (G)	-	-	-	-	-	-
97	<i>Entb. asburiae</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, (G)	-	-	-	-	-	-
Original atypical reaction in EC broth with MUG: gas, no fluorescence and no observable growth													
9	<i>K. pneumoniae</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR	GR, (G)	GR	NMG	NMG	NMG
Original atypical reactions in EC broth with MUG: growth and gas, but no fluorescence													
18	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	MG	MG	MG
19	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	MG	MG	MG
20	<i>K. pneumoniae</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	MG	MG	MG
36	<i>Entb. asburiae</i>	GR, (G)	GR, (G)	GR, (G)	GR, G	GR, G	GR, G	GR	GR	GR	NMG	NMG	NMG
Original typical reactions in EC broth with MUG: growth, gas and fluorescence													
54	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR,(G),F	GR, G, F	GR, G, F	MG	MG	MG
55	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR,(G),F	GR,(G),F	GR, G, F	MG	MG	MG
57-1	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG
57-2	<i>Entb. asburiae</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	-	-	MG	NMG	NMG
60	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG

**Table 8.11 continued**

Table 8.11 continued

Isolate	Identity	Reaction in LST			Reaction in BGLB			Reaction in EC with MUG			Reaction on L-EMB			
Original typical reactions in EC broth with MUG: growth, gas and fluorescence														
61	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG	
62	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG	
63	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG	
64-1	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG	
64-2	<i>Crono. sakazakii</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, F	GR, G, F	GR, G, F	MG	NMG	NMG	
65	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG	
66	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR,(G),F	GR, (G),F	GR,(G),F	MG	MG	MG	
67	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	MG	MG	MG	
68	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG	
71	<i>Entb. asburiae</i>	GR, G	GR, G	GR, G	GR	GR	GR	-	-	-	-	-	-	
73-1	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG	
73-2	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	MG	MG	MG	
73-3	<i>Prot. mirabilis</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	NMG	MG	
74	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG	
79	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG	
82	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR	MG	MG	MG	
84-1	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG	
84-2	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	MG	MG	MG	
85	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG	
88	<i>Entb. asburiae</i>	GR, G	GR, G	GR, G	GR	GR	GR	-	GR, G, F	-	-	MG	-	
89	<i>S. marcescens</i>	GR	GR	GR	GR	GR	GR	-	-	-	-	-	-	
90	<i>K. pneumoniae</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	NMG	NMG	NMG	

<sup>‡</sup>Reactions in brackets indicate an unacceptably weak reaction

GR: growth (turbidity), G: gas production, F: fluorescence, MG: metallic green colonies, NMG: "not metallic green" colonies, - : no reaction detected

### Isolates originally producing typical reactions in EC broth with MUG

It can be seen in Table 8.11 that of the 27 isolates which were originally isolated from typical EC broth with MUG, nine (33.3%) failed to produce the same typical reaction in isolation. Five of these isolates (57-2, 71, 88, 89 and 90) are not members of *E. coli*, and were probably isolated from a tube where they were co-occurring with an organism, or organisms, which produced the typical reaction. The theory that accidental co-occurrence resulted in subsequent transference is supported by the observation that three of these non-*E. coli* isolates, which exhibited anaerogenesis in EC broth with MUG, also failed to produce gas in BGLB broth (isolates 71 and 88) or in LST as well as BGLB broth (isolate 89) when tested individually. It is assumed, therefore, that these organisms were inadvertently transferred during the “normal” MTF with organisms which did produce a typical reaction in the medium. In addition, the failure of isolate 57-2 to produce any reaction in EC broth with MUG also agrees with this explanation, since it was isolated from co-habitation with a strain of *E. coli* (57-1) capable of growing and producing gas and fluorescence in EC broth with MUG. It is also possible that these organisms could have participated synergistically in the reaction by contributing an incomplete set of reactions, while the incomplete, or complete, reactions of another organism complemented these to result in a full typical reaction. However, the five isolates failed to produce metallic green colonies on L-EMB agar, with three not growing on the agar. Therefore, in isolation, these organisms are not a large risk for over-estimation of *E. coli*.

The remaining four organisms which failed to reproduce their original typical reactions in EC broth with MUG are strains of *E. coli*. The failure was due to a lack of fluorescence in the medium. As with the previous group of isolates, it is highly likely that these organisms were isolated from tubes where another organism was either wholly or partially responsible for the typical reaction that was produced. In this case, the organism involved in synergy would only need to produce fluorescence from MUG to complete the reaction. Isolate 73-2, for instance, was isolated from co-occurrence with another strain of *E. coli* as well as *Proteus mirabilis*, which both individually produced typical reactions in EC broth with MUG. Fortunately, the four previously mentioned isolates produced metallic green colonies on L-EMB agar, which would have resulted in an amendment of the under-estimation by EC broth with MUG *in situ*.

### Isolates originally producing atypical reactions in EC broth with MUG

#### *Growth, fluorescence and no gas*

Isolates 6, 7, 69 and 70, originally isolated from EC broth with MUG where they grew and produced fluorescence, but failed to produce gas, show mostly expected results. The two *E. coli* isolates exhibited the same atypical reaction as their *in situ* reaction. It was interesting to note, however, that these organisms were also fully anaerogenic in both LST and BGLB broths. Therefore, these organisms are very probably true anaerogenic strains of *E. coli*. Their reaction on L-EMB agar was the production of metallic green colonies, which further confirms their identification as *E. coli*. It is

doubtful, however, whether the typical reaction on L-EMB agar would result in their enumeration, since these organisms would probably not even be transferred from LST to BGLB broth unless they occurred with other aerogenic coliforms. Therefore, this could potentially result in an under-estimation of *E. coli* strains with this profile.

Isolates 69 and 70, both *Enterobacter asburiae*, failed to produce individual reactions in EC broth with MUG or on L-EMB agar. The presence of these isolates in the original EC broth with MUG tubes can be attributed to their typical reactions in both LST and BGLB broths when tested in isolation. Their inability to produce a reaction in EC broth with MUG, however, indicates that other organisms capable of growth, or fluorescence, or both; were co-habiting with these isolates during the original isolation.

#### *Growth, no fluorescence and no gas*

The isolates originally obtained from EC broth with MUG which exhibited growth but no gas or fluorescence, are four coliforms (8, 72, 94 and 97) and one strain of *Bacillus pseudofirmus* (37). Isolate 8 was the only isolate which could reproduce its original atypical reaction, and resulted in mucoid, light purple colonies on L-EMB agar. The *B. pseudofirmus* isolate showed both growth and gas production when it was inoculated individually, and produced dark purple colonies on L-EMB agar. Since both these isolates produced typical reactions in LST and BGLB broths, and would consequently reach the EC broth step, it is encouraging to observe that any resultant false positive *E. coli* enumeration would be amended by the results from L-EMB agar. The false positive enumeration of coliforms by *B. pseudofirmus*, however, would not have been detected and consequently corrected. The reason for the presence of this organism in LST, BGLB and EC broths remains unclear. It is especially strange, since some constituents of the media, such as bile in BGLB and EC broths, are specifically included to exclude Gram positive organisms. Moreover, this isolate was one of very few of the total number of isolates which did not produce a high identification with MALDI-TOF MS (best match=1.673), even after repeated analysis. Therefore, the likelihood that this isolate is a coliform which is in symbiotic co-habitation with *Bacillus* cannot be discounted. Further analyses such as sequencing of its genome, or parts thereof, is needed to prove or disprove this.

The three remaining isolates did not produce any reaction in either EC broth with MUG or L-EMB agar when they were inoculated separately. Therefore, these coliforms were correctly transferred from BGLB, but were presumably accidentally isolated from an atypical reactions caused by other unidentified bacteria. This reiterates the fact that the sampling procedure for isolation took the form of a grab sample, and the reaction from which an isolate originates is not necessarily attributable to the organism. This phenomenon, although troublesome for the isolation technique, holds much graver implications for the assay itself: the “sampling” for isolation is nearly identical to the “sample” drawn from one broth to inoculate into the next during the MTF. The

possibility of “missing” the intended test organism with this sampling procedure could result in further under-estimations of both coliforms and *E. coli* by MTF.

#### *Gas, no growth and no fluorescence*

Only one isolate was obtained from this atypical reaction in EC broth with MUG: isolate 9; identified as *Klebsiella pneumoniae*. Since the production of gas without growth of the organism is impossible, this observation was possibly made due to a negligible biomass small enough to be indiscernible from the normal level of turbidity in EC broth with MUG. The results in Table 8.11 are in agreement with this as it produced growth when it was individually inoculated into EC with MUG, and the isolate should be reassigned to the atypical reaction growth, gas and no fluorescence.

Interestingly, little or no gas was produced by the organism when it was inoculated into EC broth with MUG individually. The production of gas in the original reaction must therefore have been attributable to another organism in the medium. The organism is not, however, anaerogenic: it grew and produced considerable gas in both LST and BGLB broths.

Encouragingly, in addition to its incomplete reaction in EC broth with MUG, the isolate lacks the ability to produce metallic green colonies on L-EMB agar. Instead, it produces mucoid, light pink colonies. The atypical reactions in both these steps would exclude the organism from enumeration as *E. coli*, and it therefore poses a low methodological risk for over-estimation.

#### *Growth, gas and no fluorescence*

The final group of atypical isolates from EC broth with MUG originally resulted in growth and gas, but no fluorescence. Three isolates (18, 19 and 20) reproduced their initial atypical reaction when they were individually tested. Additionally, these organisms all produced typical reactions in LST and BGLB broths, and grew as metallic green colonies on L-EMB agar. If MUG is included in the assay, isolate 20 (re-identified as *E. coli* by molecular method) would fail to fluoresce and remain undetected though the MTF method. In contrast, isolates 18 and 19 (both *E. coli*) would not be detected by the MTF, unless L-EMB agar was used to confirm all results by EC broth with MUG. These concepts introduce the dualistic nature of MUG in this assay: while its inclusion may prevent the over-estimation of *E. coli* in some instances; it may concurrently cause an under-estimation of *E. coli* strains without  $\beta$ -D-glucuronidase.

Isolate (36) could only produce growth in EC broth with MUG when it was inoculated individually. Since this organism produced typical reactions in both LST and BGLB broths, this could not be attributed to acquired anaerogenesis. It is more likely that this organism, as many others, was isolated from co-occurrence with an organism producing gas in EC broth with MUG. The lack of gas production would exclude the organism from *E. coli* enumeration even if MUG was omitted from the assay, and the light purple colonies it produces on L-EMB agar would confirm this ruling.

The results of individual contributions by organisms originally from L-EMB are shown in Table 8.12, along with their identifications as determined by MALDI-TOF MS.

**Table 8.12** Individual reactions in MTF media elicited by isolates from L-EMB agar<sup>†</sup>

Isolate	Identity	Reaction in LST			Reaction in BGLB			Reaction in EC with MUG			Reaction on L-EMB		
Original atypical reactions on L-EMB agar: dark purple colonies													
1	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, F	GR, F	GR, F	MG	MG	MG
45	<i>E. coli</i>	GR, (G)	GR	GR, (G)	GR, G	GR, G	GR, G	GR, G, F	GR, F	GR, F	NMG	NMG	NMG
Original atypical reactions on L-EMB agar: mucoid pink													
3-1	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	NMG	NMG	NMG
3-2	<i>K. pneumoniae</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR	GR	GR	NMG	NMG	NMG
16	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG
32	<i>Entb. asburiae</i>	GR, (G)	GR, (G)	GR, (G)	GR, G	GR, G	GR, G	GR	GR	GR, G	NMG	NMG	NMG
Original atypical reactions on L-EMB agar: mucoid purple													
2	<i>K. pneumoniae</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR	GR	GR, G	NMG	NMG	NMG
81-1	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	NMG	NMG	NMG
81-2	<i>K. pneumoniae</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR	GR	GR	NMG	NMG	NMG
93	<i>Entb. kobei</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	-	NMG	NMG	-
Original typical reactions on L-EMB agar: metallic green colonies													
31	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, G, F	GR, G, F	MG	MG	MG
49	<i>K. pneumoniae</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	NMG	NMG	NMG
52	<i>E. coli</i>	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G	GR, G, F	GR, (G),F	GR,(G),F	MG	MG	MG

<sup>†</sup>Reactions in brackets indicate an unacceptably weak reaction

GR: growth (turbidity), G: gas production, F: fluorescence, MG: metallic green colonies, NMG: "not metallic green" colonies, - : no reaction detected



These results show discrepancies which can be observed in Table 8.12 once again illustrate the error which can be brought about through the “grab sample” approach. It is clear from Table 8.12 that some organisms which were originally isolated from atypical colonies on L-EMB produced typical reactions on this medium during the individual MTF analysis, and vice versa. This is presumably attributable to isolation of the incorrect organism from a mixed bacterial culture which appeared visually homogeneous on L-EMB agar.

The results for the ten atypical organisms originally isolated from L-EMB agar, presented in Table 8.12, show that eight (80.0%) remained atypical when they were tested individually. Isolate 45 reproduced its dark purple colonies on L-EMB agar. Colonies of isolates 3-1, 3-2 and 32 remained pink on L-EMB, but only 3-2 retained the mucoid character which is often associated with *Klebsiella pneumoniae*. The presence of mucoid colonies in the initial co-culture of isolates 3-1 and 3-2 is probably attributable to the influence of this organism, and isolate 3-1 was quite possibly never able to produce mucoid colonies. The reason for the absence of mucoid colonies in the individually assayed isolate 32 is not clear. It is possible that repeated purifying, cryogenic storage, or sub-culturing resulted in loss of the ability to produce exopolysaccharides.

Isolates 2, 81-1, 81-2 and 93 originally produced mucoid purple colonies on L-EMB agar. Isolate 2, 81-2 and 93 reproduced the reaction in the individual assay, but colonies of isolate 81-1 were described during the individual MTF as mucoid pink on L-EMB agar. It is possible that the ability of isolate 81-1 to produce purple colonies had been lost during various sub-culturing and preservation steps. However, all four isolates retained their mucoid character, an attribute often found in coliforms from environmental sources.

Two originally atypical isolates (1 and 16), identified as *E. coli*, produced metallic green colonies when they were individually tested. Although it is uncertain why this was observed, it is possible that organisms in the initial EC broth with MUG reaction had some inhibitory effect on these two strains and that subsequent culturing had restored the ability of the organisms to produce metallic green colonies. Conversely, it is also of interest to note that the three remaining *E. coli* strains isolated from atypical reactions did not produce metallic green colonies individually. It is possible that these organisms are the exception to the rule, but their proportion here (42.9% of all *E. coli* isolated from L-EMB) suggests that metallic green colonies on L-EMB agar may not be exclusively indicative of *E. coli*.

All the atypical isolates were able to produce typical reactions in both LST and BGLB broths; an expected result, since these organisms are coliforms. However, only three of these isolates (3-1, 16 and 81-1, all *E. coli*) were able to produce typical reactions in EC broth with MUG. This is surprising, since all organisms on L-EMB agar should have been transferred from typical EC broth with MUG reactions. This confirms, once again, that the method of transfer may not be very effective at transferring the intended organism(s). Irrefutable evidence of inadvertent transference is the presence of isolate 81-2 on L-EMB agar. Since this organism was isolated from co-culture with a strain of *E. coli* on L-EMB agar, it must have been transferred, together with the

*E. coli*, from EC broth with MUG. Fortunately the organism would not have been enumerated as *E. coli* due to its production of NMG colonies on L-EMB agar. However, the co-occurring isolate 81-1, which produced a perfect reaction in EC broth with MUG, also failed to produce typical metallic green colonies on L-EMB agar and would, therefore, not have been enumerated either.

Interestingly, the remaining two *E. coli* strains isolated from atypical colonies (1 and 45) did not produce typical reactions in EC broth with MUG. As shown in Table 8.12, both these organisms grew and fluoresced, but failed to produce gas. This failure cannot be attributed to anaerogenesis, since both these organisms produced gas in LST and BGLB broths. The failure to produce gas in EC broth with MUG cannot be attributed to suppression by other organisms either, since these isolates were tested individually. Another possible explanation for this strange reaction is that these organisms are sensitive to some compound found in EC broth. However, the composition of the broth remained constant for the initial MTF and the individual assay, and since these isolates were originally found on L-EMB agar they presumably produced typical reactions in EC broth with MUG. Therefore, chemical sensitivity could not explain the loss of gas-producing ability. The only remaining explanation is the co-occurrence with an organism capable of producing gas in the original EC broth with MUG, which is in support of the theory of synergistic reactions within the MTF method.

Two (31 and 52) isolates from L-EMB agar which was originally obtained from typical colonies exhibited metallic green colonies when they were tested individually. Both of the organisms were also identified as *E. coli*. Isolate 49, however, was identified as *Klebsiella pneumoniae* and failed to reproduce metallic green colonies when it was tested individually by MTF, producing dark purple colonies instead. It is possible that *E. coli* could have initially been present in co-culture with this isolate, despite the colonies being visually homogeneous, in which case the *E. coli* could have been responsible for the metallic green appearance. These metallic green colonies may have served to mask the NMG colonies of *Klebsiella pneumoniae*, which were observed during the individual MTF.

#### **8.4.2 Classification of risk for under- or overestimation with MTF**

The risk classification, which was done using the results obtained in the previous section, was designed to determine the risk for either under- or over-estimation posed by an individual isolate. Since the chances of finding one organism per MTF tube is highly unlikely, this is not a reflection of the reality *in situ*, but rather an assessment of the potential of an organism to adversely influence the enumeration done by MTF.

##### **Methodological risk classification for under- and over-estimation of coliforms**

The methodological risk classification for under-estimation of coliforms was done by assessing all coliform isolates (including *E. coli*) according to the criteria in Table 8.1. The production of very little gas by an organism, denoted in tables 8.9 to 8.12 by (G) was considered a negative gas

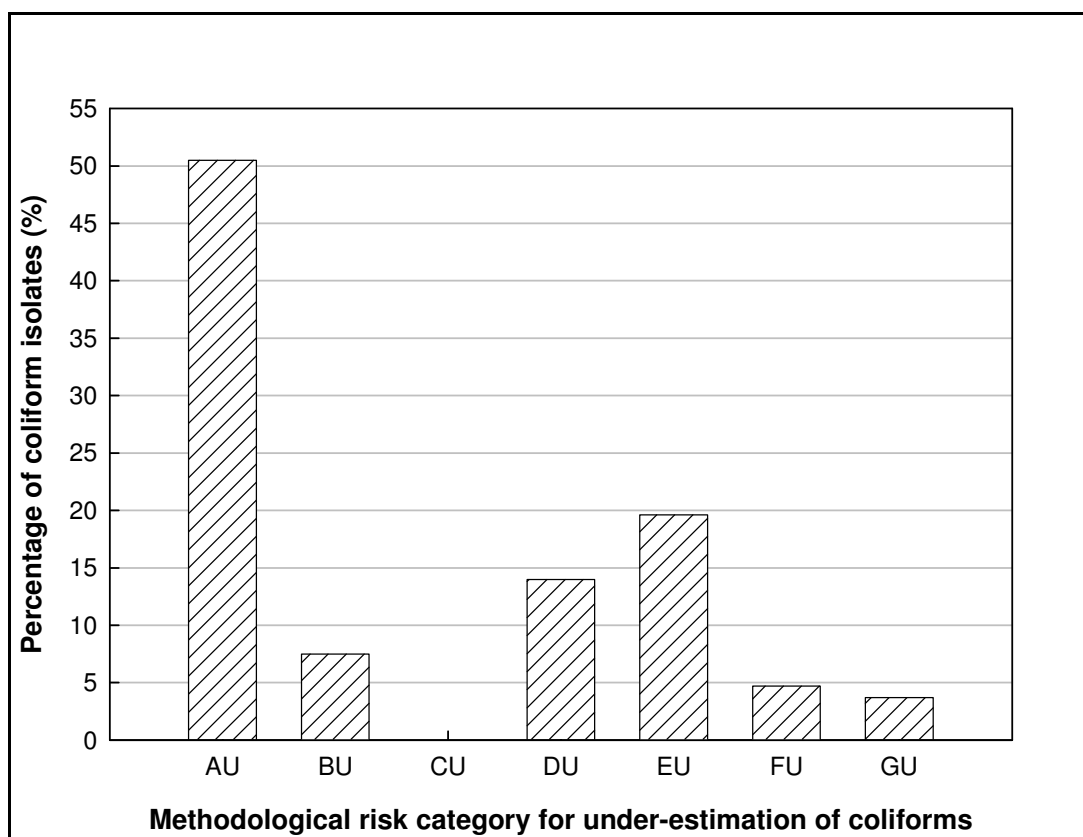
reaction for the purpose of the risk classification. Four organisms were excluded from the under-estimation classification, since they did not belong to the coliform group. These organisms are isolates 4, 21, 26 and 37. The coliform isolates, their MALDI-TOF MS identity, and their risk category for the under-estimation of coliforms are given in Table 8.13.

**Table 8.13** Individual isolates and their risk category for the under-estimation of coliforms

Isolate	Identity	Risk	Isolate	Identity	Risk	Isolate	Identity	Risk
1	<i>E. coli</i>	AU	39	<i>H. alvei</i>	DU	73-1	<i>E. coli</i>	AU
2	<i>K. pneumoniae</i>	AU	40	<i>K. oxytoca</i>	AU	73-2	<i>E. coli</i>	AU
3-1	<i>E. coli</i>	AU	41	<i>Entb. cloacae</i>	DU	73-3	<i>Prot. mirabilis</i>	AU
3-2	<i>K. pneumoniae</i>	AU	42	<i>Entb. asburiae</i>	EU	74	<i>E. coli</i>	AU
5	<i>Entb. aerogenes</i>	DU	43	<i>Entb. asburiae</i>	EU	75	<i>R. ornithinolytica</i>	EU
6	<i>E. coli</i>	EU	44	<i>Prov. rettgeri</i>	EU	79	<i>E. coli</i>	AU
7	<i>E. coli</i>	EU	45	<i>E. coli</i>	DU	80	<i>Cit. freundii</i>	DU
8	<i>K. pneumoniae</i>	AU	46	<i>K. pneumoniae</i>	DU	81-1	<i>E. coli</i>	AU
9	<i>K. pneumoniae</i>	AU	47	<i>Cit. braakii</i>	DU	81-2	<i>K. pneumoniae</i>	AU
11-1	<i>Cit. braakii</i>	AU	48-1	<i>E. coli</i>	AU	82	<i>E. coli</i>	AU
11-2	<i>M. morganii</i>	EU	48-2	<i>S. marcescens</i>	BU	84-1	<i>E. coli</i>	AU
12	<i>Cit. braakii</i>	DU	49	<i>K. pneumoniae</i>	AU	84-2	<i>E. coli</i>	AU
13-1	<i>E. coli</i>	AU	50	<i>Entb. asburiae</i>	DU	85	<i>E. coli</i>	AU
13-2	<i>Prov. alcalifaciens</i>	AU	51	<i>R. ornithinolytica</i>	AU	86	<i>R. ornithinolytica</i>	FU
14	<i>Prov. alcalifaciens</i>	GU	52	<i>E. coli</i>	AU	87	<i>Entb. kobei</i>	DU
15	<i>K. pneumoniae</i>	GU	53	<i>K. pneumoniae</i>	BU	88	<i>Entb. asburiae</i>	BU
16	<i>E. coli</i>	AU	54	<i>E. coli</i>	AU	89	<i>S. marcescens</i>	EU
17	<i>Entb. asburiae</i>	DU	55	<i>E. coli</i>	AU	90	<i>K. pneumoniae</i>	AU
18	<i>E. coli</i>	AU	56	<i>Entb. cloacae</i>	AU	92	<i>Cit. braakii</i>	FU
19	<i>E. coli</i>	AU	57-1	<i>E. coli</i>	AU	93	<i>Entb. kobei</i>	AU
20	<i>K. pneumoniae</i>	AU	57-2	<i>Entb. asburiae</i>	AU	94	<i>Cit. freundii</i>	BU
22	<i>M. morganii</i>	EU	59	<i>K. pneumoniae</i>	BU	95	<i>E. coli</i>	EU
23	<i>M. morganii</i>	AU	60	<i>E. coli</i>	AU	97	<i>Entb. asburiae</i>	AU
24-1	<i>Entb. asburiae</i>	DU	61	<i>E. coli</i>	AU	98	<i>Prov. stuartii</i>	AU
24-2	<i>M. morganii</i>	EU	62	<i>E. coli</i>	AU	99	<i>Prot. vulgaris</i>	FU
25	<i>Entb. radicincitans</i>	EU	63	<i>E. coli</i>	AU	100	<i>Prot. mirabilis</i>	EU
27	<i>K. pneumoniae</i>	EU	64-1	<i>E. coli</i>	AU	101	<i>Prot. vulgaris</i>	EU
28	<i>K. pneumoniae</i>	FU	64-2	<i>Cr. sakazakii</i>	AU	102	<i>Prot. vulgaris</i>	EU
29	<i>R. ornithinolytica</i>	AU	65	<i>E. coli</i>	AU	103	<i>Prot. mirabilis</i>	EU
30	<i>S. marcescens</i>	EU	66	<i>E. coli</i>	AU	104	<i>Prov. stuartii</i>	GU
31	<i>E. coli</i>	AU	67	<i>E. coli</i>	AU	105	<i>Prot. mirabilis</i>	GU
32	<i>Entb. asburiae</i>	DU	68	<i>E. coli</i>	AU	106	<i>Prot. mirabilis</i>	AU
34	<i>Entb. asburiae</i>	DU	69	<i>Entb. asburiae</i>	BU	107	<i>Prot. vulgaris</i>	EU
35	<i>S. marcescens</i>	FU	70	<i>Entb. asburiae</i>	BU	108	<i>K. pneumoniae</i>	AU
36	<i>Entb. asburiae</i>	DU	71	<i>Entb. asburiae</i>	BU	109	<i>Prov. stuartii</i>	EU
38	<i>K. oxytoca</i>	AU	72	<i>Entb. kobei</i>	EU			

The AU category in the table is representative of a reaction which does not result in any under-estimation of coliforms, since its definition is the production of growth and gas in LST and BGLB broths. The majority (50.5%) of the coliforms were classified to this category, including 30 of the 34 *E. coli* isolates. Therefore, these isolates would be correctly enumerated as presumptive and confirmed coliforms in LST and BGLB broths, respectively. Furthermore, this classification indicates that of the coliform isolates which were obtained throughout the MTF method, more than half would cause no under-estimation in the enumeration of coliforms.

The remaining 49.5% of isolates were distributed among five other categories. No isolates were classified in category CU, which described the production of growth and gas in LST broth, but a failure to produce growth and gas in BGLB broth. Except for category AU, it is difficult to determine from the table which other categories were dominant in the classification of the isolates. Therefore, the results are presented visually in Fig. 8.1.



**Figure 8.1** Distribution of all coliform isolates (n=107) among risk categories for the under-estimation of coliforms

It is evident from Fig. 8.1 that more than half of the coliform isolates were categorised in AU. This distribution of isolates among the categories is encouraging for the accuracy of the method, especially since coliforms categorised within the “ideal” AU category represents the 50.5% of isolates tested. Despite this, the maximum risk of under-estimation of coliforms by MTF is

49.5% according to the isolates which were tested during the study. However, the proportion of isolates which occurred in the lower risk end of the histogram (the first half of the categories) was 66.8%, while the isolates occurring in the high risk end of the histogram only amounted to 33.2%.

The second and third largest categories were EU and DU, respectively. These categories describe isolates which behaved anaerogenically in either LST broth (category DU), or in LST and BGLB broths (category EU); signifying that the greatest problem relating to the under-estimation of coliforms is anaerogenicity in LST broth. Since these organisms had been cryogenically frozen and subsequently subcultured numerous times, the observed anaerogenesis is, presumably, not due to reparable injury of these coliforms and they are accepted to be true anaerogenic strains.

Category BU, which describes a typical reaction in LST broth and a subsequent anaerogenic reaction in BGLB broth, was represented by eight of the isolates. This reaffirms the observation that the most problematic factor in the under-estimation of coliforms is anaerogenesis in LST broth. When the number of isolates in category BU is compared with the number of isolates classified under category DU (15), it is clear to see that anaerogenesis in LST broth occurred at nearly double the frequency of cases of anaerogenesis in BGLB broth. The interpretation of the resulting implications, in this case, can be positive or negative. Optimistically, a higher frequency of anaerogenic reactions in LST broth would result in lower presumptive coliform enumerations while the confirmed coliform enumeration, which some would argue is of more significance, would be affected to a lesser extent. This idea, however, assumes that anaerogenic organisms in LST are transferred to BGLB to be enumerated as confirmed coliforms. This transfer of the contents of negative reactions is not standard laboratory practice due to both labour and financial constraints, and quite possibly only occurs inadvertently due to transfer with aerogenic coliforms or misinterpretation of a turbid tube as also possessing produced gas. The pessimistic view, which is in this case more true to the reality in a laboratory scenario, would see the anaerogenic coliforms in LST not being transferred to BGLB and, worse yet, not being enumerated even as presumptive coliforms.

The categories which represented the most severe risk for under-estimation were FU and GU, and comprised 5 and 4 isolates, respectively. Category FU contained isolates which could only grow in LST broth and failed to produce any positive reactions in BGLB broth. The isolates classified in this category are 28, 35, 86, 92 and 99. Three of these isolates (28, 35 and 86) were isolated from anaerogenic LST reactions. Isolates 92 and 99 were isolated respectively from atypical and typical BGLB tubes. Their failure to produce any reaction in BGLB during the individual assay alludes to their co-habitation with other isolates which may have been responsible for the positive or partially positive initial reactions. The reactions of the five isolates, irrespective of their isolation history, remains grounds for concern since these isolates all belonged to known genera in the coliform grouping and would not have been enumerated as confirmed coliforms due to their failure to produce typical reactions in BGLB broth. Even their enumeration as presumptive coliforms is highly unlikely, since these organisms failed to produce gas in LST broth. Therefore,

the only way these organisms could be enumerated as presumptive coliforms is if an error was made by the technician, by misinterpreting a turbid LST tube. The inaccurate interpretation of results could never be viewed as a positive attribute, and if these isolates were “mistakenly” correctly enumerated, there is no telling how many non-coliforms would be “mistakenly” incorrectly enumerated by the same bias towards error.

The highest risk category, GU, was associated with failure to produce any positive reaction in LST or BGLB broths. Four coliform isolates (14, 15, 104 and 105) resorted in this category. Isolates 14 and 104 belonged to the genus *Providencia*, while isolate 105 belonged to the genus *Proteus*. It has already been theorised in a previous chapter (see section 7.4.1) that strains of these organisms were prone to anaerogenesis due to their failure to produce a positive reaction with 2-nitrophenyl- $\beta$ -D-galactopyranoside in API 20E. At closer inspection, it was found that 11 (73.3%) of the 15 *Providencia* or *Proteus* isolates were associated with some degree of anaerogenesis: either in LST broth; or BGLB broth; or in both. However, it should be noted that four isolates belonging to *Providencia* or *Proteus* were categorised in the “ideal” AU category. Furthermore, the species level of the four isolates, namely *Providencia alcalifaciens*, *Providencia stuartii* and *Proteus mirabilis* were all represented within the anaerogenic categories as well. This indicates that the observed anaerogenic trend cannot be attributed to the entire genus or species, but should be seen as a predisposition by individual members of these genera and species for anaerogenic behaviour.

The fourth isolate classified within GU was identified as *Klebsiella pneumoniae*. It is with some concern that the presence of this isolate is noted in this category, since the genus is considered to be an important member of the coliform grouping and, furthermore, forms part of the thermotolerant coliform subgroup (Leclerc *et al.*, 2001). The relative importance of the isolate should also, however, be considered: the vast majority, 64.7%, of *Klebsiella* isolates were categorised in category AU, while all other categories, except CU, were represented by 5.9% of *Klebsiella* isolates. The anaerogenic nature of some coliforms has been widely observed in Chapter 5, and the representation by *Klebsiella* minorities in these categories is not surprising. Even so, the presence of isolate 15 in a category which assigns an inability to grow or produce gas in LST and BGLB broths is problematic, not only for the enumeration of coliforms, but also if subsequent thermotolerant coliform enumerations are performed.

The minimum concentrations of all problem category isolates in the original sample, calculated from the serial dilution from which they were isolated, are given in Table 8.14. These concentrations indicate the minimum number of these problematic organisms in the sample.

**Table 8.14** Minimum concentrations of isolates assigned to risk categories for the under-estimation of coliforms

Isolate	Minimum concentration range (cfu.mL <sup>-1</sup> )	Risk	Isolate	Minimum concentration range (cfu.mL <sup>-1</sup> )	Risk	Isolate	Minimum concentration range (cfu.mL <sup>-1</sup> )	Risk
5	10 <sup>2</sup>	DU	36	10 <sup>0</sup>	DU	80	10 <sup>2</sup>	DU
6	10 <sup>0</sup>	EU	39	10 <sup>2</sup>	DU	86	10 <sup>1</sup>	FU
7	10 <sup>1</sup>	EU	41	10 <sup>1</sup>	DU	87	10 <sup>0</sup>	DU
11-2	10 <sup>0</sup>	EU	42	10 <sup>1</sup>	EU	88	10 <sup>0</sup>	BU

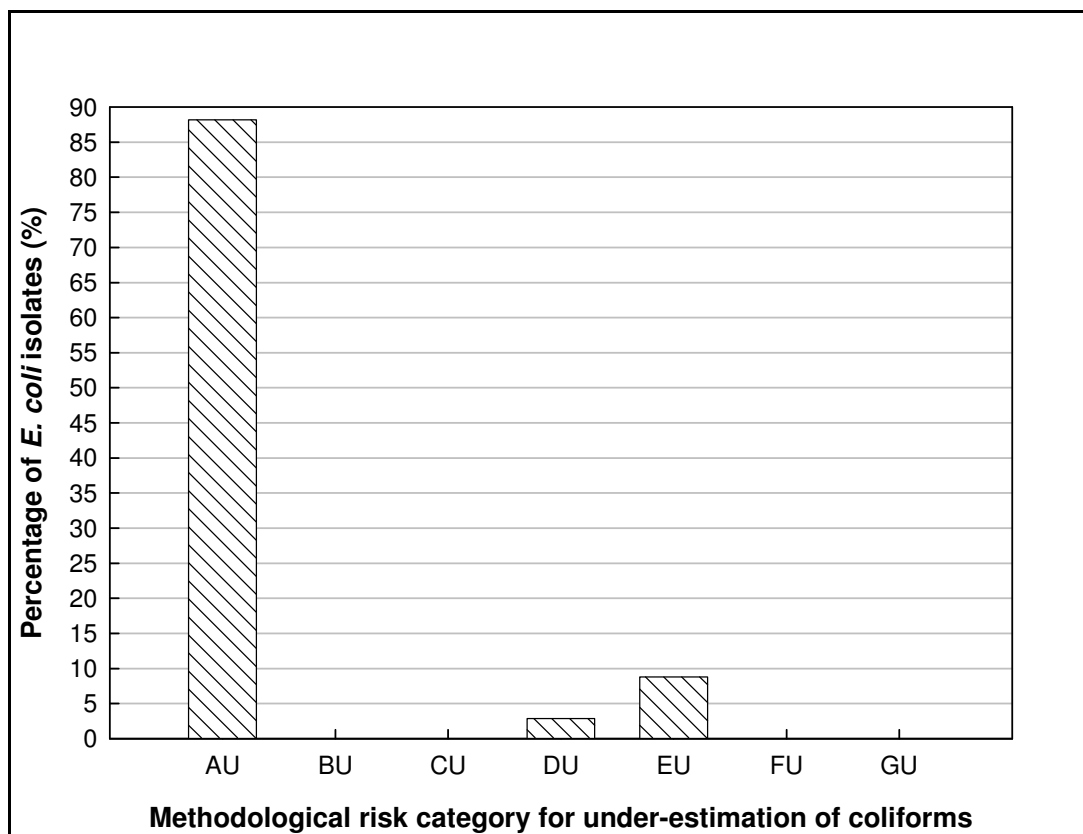
**Table 8.14 continued**

Isolate	Minimum concentration range (cfu.mL <sup>-1</sup> )	Risk	Isolate	Minimum concentration range (cfu.mL <sup>-1</sup> )	Risk	Isolate	Minimum concentration range (cfu.mL <sup>-1</sup> )	Risk
12	10 <sup>0</sup>	DU	43	10 <sup>1</sup>	EU	89	10 <sup>0</sup>	EU
14	10 <sup>0</sup>	GU	44	10 <sup>1</sup>	EU	92	10 <sup>2</sup>	FU
15	10 <sup>2</sup>	GU	45	10 <sup>0</sup>	DU	94	10 <sup>2</sup>	BU
17	10 <sup>1</sup>	DU	46	10 <sup>1</sup>	DU	95	10 <sup>2</sup>	EU
22	10 <sup>0</sup>	EU	47	10 <sup>1</sup>	DU	99	10 <sup>1</sup>	FU
24-1	10 <sup>1</sup>	DU	48-2	10 <sup>0</sup>	BU	100	10 <sup>3</sup>	EU
24-2	10 <sup>1</sup>	EU	50	10 <sup>1</sup>	DU	101	10 <sup>2</sup>	EU
25	10 <sup>3</sup>	EU	53	10 <sup>0</sup>	BU	102	10 <sup>0</sup>	EU
27	10 <sup>2</sup>	EU	59	10 <sup>1</sup>	BU	103	10 <sup>0</sup>	EU
28	10 <sup>2</sup>	FU	69	10 <sup>2</sup>	BU	104	10 <sup>0</sup>	GU
30	10 <sup>1</sup>	EU	70	10 <sup>2</sup>	BU	105	10 <sup>0</sup>	GU
32	10 <sup>0</sup>	DU	71	10 <sup>1</sup>	BU	107	10 <sup>1</sup>	EU
34	10 <sup>0</sup>	DU	72	10 <sup>2</sup>	EU	109	10 <sup>0</sup>	EU
35	10 <sup>0</sup>	FU	75	10 <sup>3</sup>	EU			

As can be seen from Table 8.14, the minimum concentrations of problematic isolates ranged from 10<sup>0</sup> to 10<sup>3</sup> cfu.mL<sup>-1</sup>. More than a third (37.7%) of these isolates had a minimum concentration of 10<sup>0</sup>, with the remaining isolates at minimum concentrations of 10, 100 or 1000 cfu.mL<sup>-1</sup>. To determine the risk posed by these isolates the concentrations should be considered with the corresponding risk category of the isolate. By this argument, isolate 75 in category EU would probably result in a larger under-estimation than isolate 104 in category GU; since the former would be present in much larger numbers. However, this is not necessarily the case: isolate 104 may well be present at 10<sup>3</sup> cfu.mL<sup>-1</sup>; since the concentrations in Table 8.14 are *minimum* concentrations based on the dilution from which the organism was isolated. It is conceded that the conclusions drawn from the minimum concentration data are extremely limited since isolates were obtained from a single decimal dilution. However, this approach illustrates further sophistication of the risk classification system which could be employed in future work.



The distribution of *E. coli* isolates, which were considered as coliforms for the risk classification relating to the enumeration of coliforms, were plotted separately. This was done to determine the methodological risk of under-estimating *E. coli* in the first steps of the MTF method, since failure to detect *E. coli* in the initial steps would result in non-transference to EC broth with MUG. The distribution of *E. coli* isolates across the methodological risk categories for the under-estimation of coliforms is given in Fig. 8.2.



**Figure 8.2** Distribution of *E. coli* isolates (n=34) among risk categories for the under-estimation of coliforms

It can be seen in Fig. 8.2 that 30 of the 34 *E. coli* isolates (88.2%) were assigned to the AU category, which was defined as no risk for the under-estimation of coliforms. Therefore, based on the isolates used in this analysis, the majority of organisms belonging to *E. coli* do not pose a risk for under-estimation of coliform numbers in MTF, with a maximum risk of 11.8% arising from the isolates tested in this study.

Of the four isolates which did not fall within this category, three were found to cause anaerogenic reactions in both LST and BGLB broths (category EU) and one was found to be incapable of producing gas in LST broth (category DU). From these results it can be concluded that the under-estimation of *E. coli* as a coliform is predominantly due to anaerogenesis either in LST broth, or in both LST and BGLB broth. This conclusion agrees with the conclusions made

based on the results for the entire group of coliform isolates. Therefore, it is important that the identification of anaerogenic coliforms as a major causative agent in the under-estimation of coliforms is kept in mind when ways of improving the MTF method are considered.

The risk classification for the over-estimation of coliforms by MTF was performed on the four non-coliform organisms which were identified from the 111 isolates which were examined. The results of this risk classification are given in Table 8.15.

**Table 8.15** Individual isolates and their risk category for the over-estimation of coliforms

Isolate	Identity	Risk	Isolate	Identity	Risk
4	<i>A. hydrophila</i>	CO	26	<i>B. pseudofirmus</i>	EO
21	<i>M. luteus</i>	AO	37	<i>B. pseudofirmus</i>	GO

It is clear from the small sample set in Table 8.15 that only very limited conclusions can be drawn from four isolates shown. One isolate, *Micrococcus luteus* (21), did not pose any risk for the over-estimation of coliforms since it was categorised in category AO, which describes the failure to grow or produce gas in LST (which automatically excludes its inoculation in BGLB broth from the MTF assay). Therefore, *M. luteus* is presumed incapable of a reaction in the MTF media, especially since this organism is not a coliform and does not even have morphology in common with the coliforms. However, this was the only strain of *M. luteus* which was obtained and examined, which largely limits the greater applicability of the latter conclusion.

Isolate 4, *Aeromonas hydrophila*, posed a slight risk for the over-estimation of coliforms in the MTF by causing anaerogenic growth reactions in both LST and BGLB. It has been stated previously that tubes examined during an *in situ* MTF assay should be carefully examined, since the absence of gas is more difficult to detect in turbid tubes. If this is the case, isolate 4 could cause over-estimation of coliform numbers at both the presumptive and confirmatory stage of MTF.

Two isolates which were identified by MALDI-TOF MS as *Bacillus pseudofirmus* (26 and 37) caused varied reactions in LST and BGLB broths: while isolate 26 could only produce a typical reaction in LST broth, isolate 37 could produce typical reactions in both LST and BGLB broths. Consequently, isolate 26 would be enumerated as a presumptive coliform, but would be rejected as a confirmed coliform in BGLB broth. Therefore, its risk for the over-estimation of coliforms, although admittedly higher than that of isolate 4, is still small. Isolate 37, however, poses the maximum risk possible for the over-estimation of coliforms. This organism would be enumerated as a presumptive coliform in LST broth and would be confirmed as such in BGLB broth. The detection of a species of *Bacillus* which possesses the ability to ferment lactose has been described by Ordal & Halvorson (1939), who identified that *B. colimutabile* produced lactose fermenting variants. Since these two isolates (26 and 37) were identified as the same species, it is clear that strains of the same species do not necessarily pose the same risk for over-estimation, or

any incorrect enumeration, in the MTF method. This was also illustrated in the discussion relating to the risk of under-estimation of coliforms, where varied reactions were observed within the genus *Klebsiella*. It should be kept in mind, however, that both of these isolates had low identification values with MALDI-TOF MS (1.740 and 1.673 for isolate 26 and 37, respectively), and may have been misidentified.

The minimum concentration for each of the three isolates occurring in problematic risk categories was  $10^1$  cfu.mL<sup>-1</sup>,  $10^2$  cfu.mL<sup>-1</sup> and  $10^0$  cfu.mL<sup>-1</sup> for isolates 4, 26 and 37, respectively. If these concentrations are a reflection of the true concentrations of the isolates in the original sample, isolate 26 would have been the most problematic due to the number of organisms which posed a category EO methodological risk.

At this point, it is important to mention the very small proportion of isolates which did not belong to the coliform grouping. Although the isolation procedure used in Chapter 5 has an intrinsic bias towards atypical reactions and the sampling took on a “grab sample” format; which has been shown in a previous chapter to sample indiscriminately and, sometimes, miss the intended target organism; this should not have resulted in lower numbers of non-coliforms. On the contrary, grab sampling LST and BGLB broth tubes with atypical reactions should have skewed the numbers towards non-coliforms, since these organisms are expected to show erratic growth in these media. Since this was not the case, it is assumed that non-coliforms in the sample simply could not grow in the selective environment created by the MTF media and that this is the reason for the low isolation frequency of non-coliforms. Following this reasoning, it can be concluded that the over-estimation of coliforms due to non-coliforms is not of great concern due to the effective suppression of non-coliforms by the media. Furthermore, microbiological methods often have some degree of error in their measurement, it is always preferable to have a method which errs on the side of caution (in this case, over-estimation) in the assessment of risk.

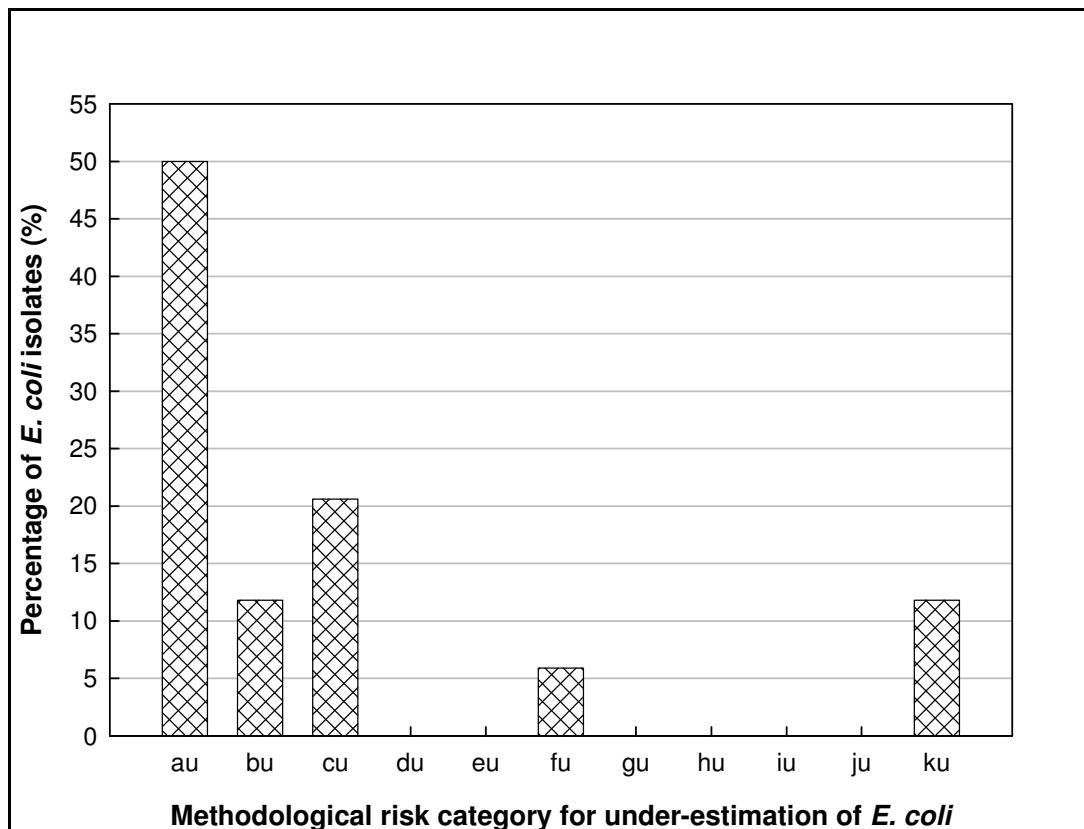
#### Methodological risk classification for under- and over-estimation of *E. coli*

Thirty-four *E. coli* isolates were evaluated for their contribution towards the methodological risk for under-estimation of *E. coli*. These isolates and the methodological risk category assigned to each are given in Table 8.16.

**Table 8.16** Individual isolates and their risk category for the under-estimation of *E. coli*

Isolate	Risk	Isolate	Risk	Isolate	Risk	Isolate	Risk	Isolate	Risk	Isolate	Risk
1	bu	18	cu	54	au	63	au	73-1	au	84-1	au
3-1	fu	19	cu	55	bu	64-1	au	73-2	cu	84-2	cu
6	ku	31	au	57-1	au	65	au	74	au	85	au
7	ku	45	ku	60	au	66	bu	79	au	95	ku
13-1	au	48-1	cu	61	au	67	cu	81-1	fu		
16	au	52	bu	62	au	68	au	82	cu		

The number of isolates in Table 8.16 which were assigned to the “no risk” category au, which describes an organism which elicits gas, growth and fluorescence in EC broth with MUG and metallic green colonies on L-EMB agar, is 17 or 50.0% of the *E. coli* isolates. Of these organisms, 14 (82.4%) were originally isolated from typical EC broth with MUG reactions. There was one isolate (13-1) which was isolated from an atypical LST reaction. This atypical reaction in the first step would most likely have lead to the organism not being enumerated as a coliform or transferred to EC broth with MUG for enumeration as *E. coli*. Interestingly, when this isolate was individually inoculated into the media of the MTF method, it produced gas in LST broth. This is further affirmation for the observation made in this study and by various other workers (Evans *et al.*, 1981; Edberg & Edberg, 1988) that the presence of heterotrophic bacteria in the sample can suppress the ability of coliforms to produce gas from lactose. The two remaining isolates were both isolated from L-EMB agar, where one (16) resulted in a typical reaction and the other (31) an atypical reaction. Since these organisms were isolated from L-EMB agar, they were most likely present in a tube of EC broth with MUG with a typical reaction. To better visualise the distribution of the isolates among the other ten categories for the risk of under-estimation of *E. coli*, the percentages per category are presented as Fig. 8.3.



**Figure 8.3** Distribution of *E. coli* isolates (n=34) among risk categories for the under-estimation of *E. coli*

As can be seen from Fig. 8.3 category au represented 50.0% of the *E. coli* isolates. Therefore, with the isolates used in this analysis, the maximum methodological risk of any degree of under-estimation of *E. coli* is 50.0%. Another 32.4% of the isolates make up the two lowest risk categories for under-estimation. Four isolates occurred in category bu (growth, fluorescence but no gas in EC broth with MUG; metallic green colonies on L-EMB agar). This is considered the lowest risk for under-estimation, since the lack of gas in an inverted Durham tube inside a turbid test tube could be accidentally overlooked, especially in the case of inexperienced analysts. Seven isolates occurred in category cu, which describes gas, growth and the absence of a fluorescent reaction in EC broth with MUG and subsequent metallic green colonies on L-EMB agar. As discussed under the methodology section, this was seen as a higher risk for under-estimation, since the absence of fluorescence under the UV light is unlikely to be missed. However, if the contents of the EC broth with MUG tubes were streaked out, irrespective of their anomalous reactions, and revealed metallic green growth on L-EMB agar the enumeration would be confirmed in the case of initial inclusion of these tubes and amended in the case of initial exclusion of these tubes. This is the case for all categories up to category eu, but it necessitates the streaking out of all EC broth with MUG tubes, both typical and atypical. The increased labour and media requirement could be easily justified by the increased sensitivity of the enumeration, but it should be kept in mind that Fig. 8.3 also shows the presence of *E. coli* isolates among categories which describe NMG colonies on L-EMB agar. In addition, the possibility of non-*E. coli* isolates resulting in metallic green colonies on L-EMB agar cannot be ignored. Such an isolate (49) was originally obtained from L-EMB agar and was identified as *Klebsiella pneumoniae* spp. *pneumoniae* (see section 5.4.12).

Category fu in Fig. 8.3 is a prime example of the dualistic role of L-EMB on the enumeration of *E. coli*. Since this category describes a perfect typical reaction by the isolate in EC broth with MUG and subsequent NMG colonies on L-EMB agar, the confirmatory agar would, in this case, cause the isolate to be incorrectly removed from the enumeration. In such a case, the omission of L-EMB agar would have been more beneficial to the enumeration accuracy of the organism. It is at this point where the distribution of the isolates becomes extremely important: the number of *E. coli* isolates which resulted in metallic green colonies on L-EMB agar was 31; while the number of *E. coli* isolates which failed to produce metallic green colonies on L-EMB agar was three. Therefore, based on the results of the *E. coli* isolates alone, L-EMB agar is a predominantly positive influence on the accuracy of the enumeration. No conclusive answer can be provided here regarding the inclusion or exclusion of L-EMB agar from the assay, since the influence of L-EMB on the over-estimation of *E. coli* numbers, as well will be discussed in the next section.

Category ku was also represented by a number of isolates. This is the worst case scenario for the under-estimation of *E. coli*, since the isolates which fall into this category were not detected by LST and/or BGLB broths, and would consequently not be assayed for *E. coli* in EC broth with

MUG. The isolates were mostly anaerogenic in the former broths, indicating that the anaerogenic nature of some coliforms can impact severely on the accuracy of the enumerations obtained by MTF. If the high likelihood of the co-habitation of these organisms with aerogenic coliforms in LST and BGLB broths is considered and it is further assumed that these isolates did reach EC broth with MUG, the isolates would be reclassified to category bu (6, 7 and 95) and category gu (45). Therefore, three of the isolates would have had a reasonable chance of being enumerated as *E. coli* if their reactions in LST and BGLB were typical.

Finally, the proportion of isolates which fell into the first half (lower risk) of the categories was 85.3% and the proportion which fell into the second half (higher risk) of the categories was 14.7%.

The minimum concentrations of all problem category *E. coli* isolates in the original sample, calculated from the serial dilution from which they were isolated, are given in Table 8.17. These concentrations indicate the minimum number of these problematic organisms in the sample.

**Table 8.17** Minimum concentrations of isolates assigned to risk categories for the under-estimation of *E. coli*

Isolate	Minimum concentration range (cfu.mL <sup>-1</sup> )	Risk	Isolate	Minimum concentration range (cfu.mL <sup>-1</sup> )	Risk	Isolate	Minimum concentration range (cfu.mL <sup>-1</sup> )	Risk
1	10 <sup>0</sup>	bu	45	10 <sup>0</sup>	ku	73-2	10 <sup>0</sup>	cu
3-1	10 <sup>1</sup>	fu	48-1	10 <sup>0</sup>	cu	81-1	10 <sup>0</sup>	fu
6	10 <sup>0</sup>	ku	52	10 <sup>0</sup>	bu	82	10 <sup>0</sup>	cu
7	10 <sup>1</sup>	ku	55	10 <sup>0</sup>	bu	84-2	10 <sup>0</sup>	cu
18	10 <sup>1</sup>	cu	66	10 <sup>0</sup>	bu	95	10 <sup>2</sup>	ku
19	10 <sup>2</sup>	cu	67	10 <sup>0</sup>	cu			

As discussed under Table 8.14, the concentrations in Table 8.17 should be considered with the risk category of each isolate. From this it can be inferred that an organism such as isolate 19 in category cu could result in larger under-estimations of *E. coli* than isolate 45 in category ku due to the numbers present in the sample. However, these inferences are based on the assumption that the minimum concentrations are true concentrations; an assumption which cannot be empirically supported or refuted here.

The presence of MUG in the *E. coli* assay, as with L-EMB, can be seen as dualistic: although it can refine the criteria for *E. coli* detection and thereby exclude some gas producing non-*E. coli* organisms, it may also exclude  $\beta$ -D-glucuronidase negative *E. coli* and increase the under-estimation of *E. coli*. To determine the extent of this phenomenon, "MUG" was excluded from the risk classification by removing the fluorescence in EC broth criteria from the assessment. The results obtained from this modified risk classification for the under-estimation of *E. coli* is given in Table 8.18.

As it can be seen in the table (on the next page), most organisms fell within the first “no risk” category Mi when MUG was removed from the assay. The number of isolates within the first category increased from 17 (50.0%) to 24 (70.6%). The seven isolates which were reclassified by the modification all originated from the previous cu category, which described gas, growth but no fluorescence in EC broth with MUG.

**Table 8.18** Individual isolates and their risk category for the under-estimation of *E. coli* in an MTF assay with no MUG

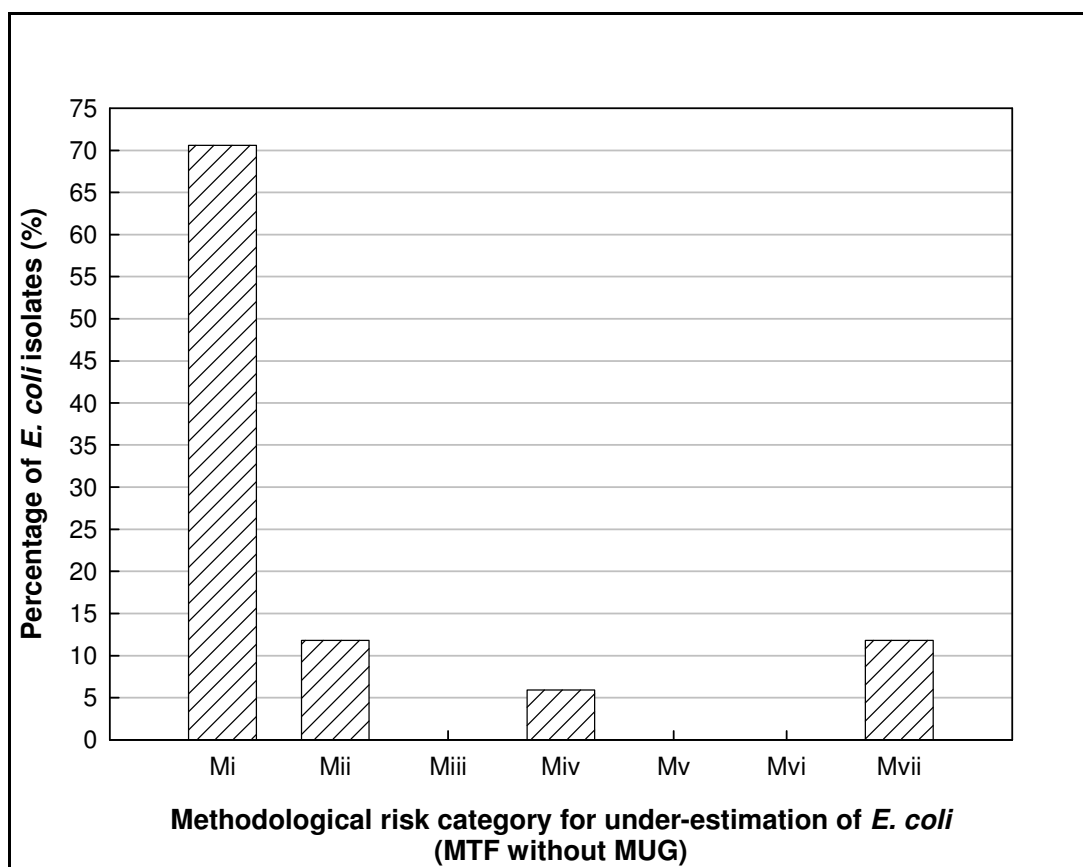
Isolate	Risk	Isolate	Risk	Isolate	Risk	Isolate	Risk	Isolate	Risk	Isolate	Risk
1	Mvii	18	Mi	54	Mi	63	Mi	73-1	Mi	84-1	Mi
3-1	Miv	19	Mi	55	Mii	64-1	Mi	73-2	Mi	84-2	Mi
6	Mii	31	Mi	57-1	Mi	65	Mi	74	Mi	85	Mi
7	Mvii	45	Mvii	60	Mi	66	Mii	79	Mi	95	Mvii
13-1	Mi	48-1	Mi	61	Mi	67	Mi	81-1	Miv		
16	Mi	52	Mii	62	Mi	68	Mi	82	Mi		

Since these isolates (18, 19, 48-1, 67, 73-2, 82 and 84-2) did not produce a fluorescent reaction with MUG, it was classified as cu in the previous risk classification. However, without the fluorescent criterion, these organisms produced full typical reactions and were reclassified into the “no risk” category. To visualise the distribution of the remaining isolates across the modified categories, the numbers per category are presented as Fig. 8.4.

As expected, the other categories remained the same, since these categories which contained isolates in Fig. 8.3 were all associated with the production fluorescence. In isolation, the increase of isolates in the first category cannot be seen as a positive, since the effect of the absence of MUG has not yet been demonstrated on the risk of over-estimation of *E. coli* by non-*E. coli* isolates. The proportion of isolates which now occurred in the first half of the categories was 85.3%, while the proportion of isolates in the second half of the categories was 14.7%.

Therefore, no change was observed in the proportions when compared to those obtained from the analysis which included MUG. In addition, it should be noted that apart from the reclassification of isolates to the first category, the proportions of isolates in metallic green and NMG categories did not change from those calculated for the initial risk classification for the under-estimation of *E. coli* either. For this reason, the effect of L-EMB agar on the assay was examined by omitting the colony growth on L-EMB criterion from the risk classification.





**Figure 8.4** Distribution of *E. coli* isolates (n=34) among risk categories for the under-estimation of *E. coli* in an MTF assay without MUG

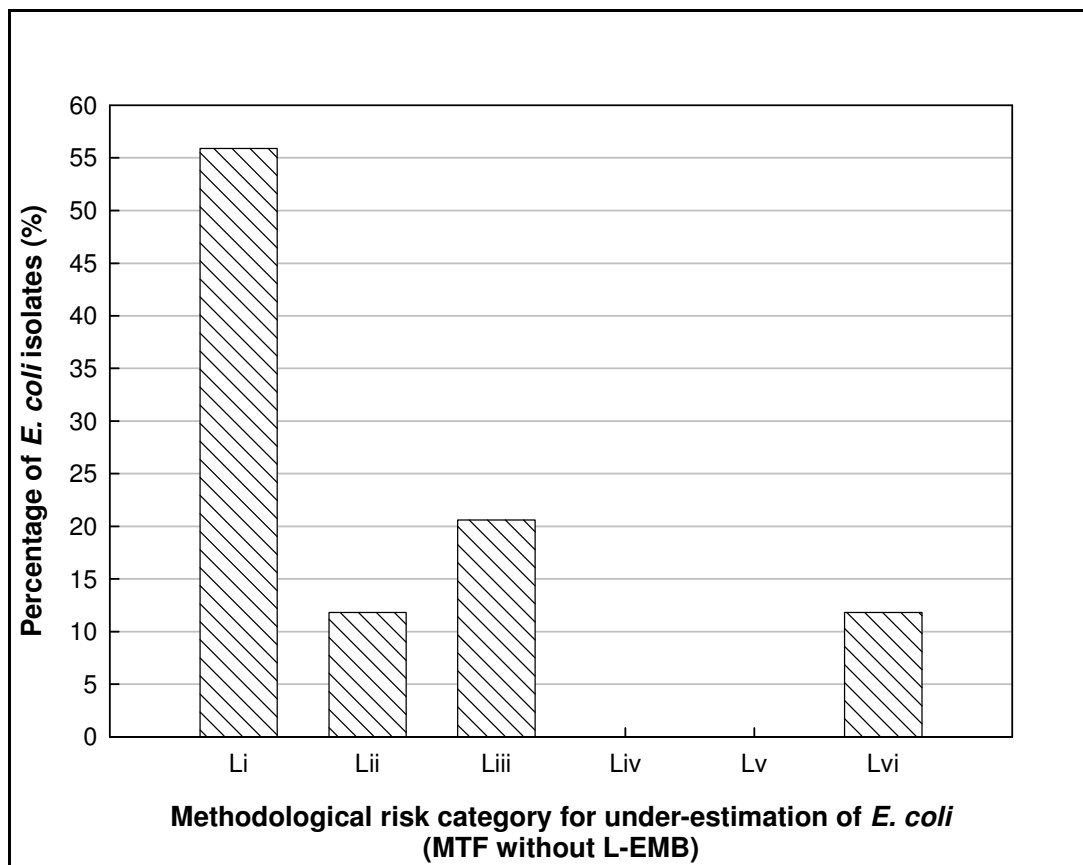
The results of the modified risk classification which omitted L-EMB from the analysis is shown in Table 8.19.

**Table 8.19** Individual isolates and their risk category for the under-estimation of *E. coli* in an MTF assay with no confirmation by L-EMB agar

Isolate	Risk	Isolate	Risk	Isolate	Risk	Isolate	Risk	Isolate	Risk	Isolate	Risk
1	Lii	18	Liii	54	Li	63	Li	73-1	Li	84-1	Li
3-1	Li	19	Liii	55	Lii	64-1	Li	73-2	Liii	84-2	Liii
6	Lvi	31	Li	57-1	Li	65	Li	74	Li	85	Li
7	Lvi	45	Lvi	60	Li	66	Lii	79	Li	95	Lvi
13-1	Li	48-1	Liii	61	Li	67	Liii	81-1	Li		
16	Li	52	Lii	62	Li	68	Li	82	Liii		

The results in Table 8.19 indicate that the number of isolates which occurred in the first “no risk” category for the under-estimation of *E. coli*, with growth L-EMB agar omitted, was 19 (55.9%). This is an increase from number of isolates in the first category for the initial risk classification, but not as large an increase as was observed through the omission of fluorescence as a criterion. The

distribution of the isolates among the first category, as well as the other categories, is presented in Fig. 8.5.



**Figure 8.5** Distribution of *E. coli* isolates (n=34) among risk categories for the under-estimation of *E. coli* in an MTF assay without confirmation on L-EMB agar

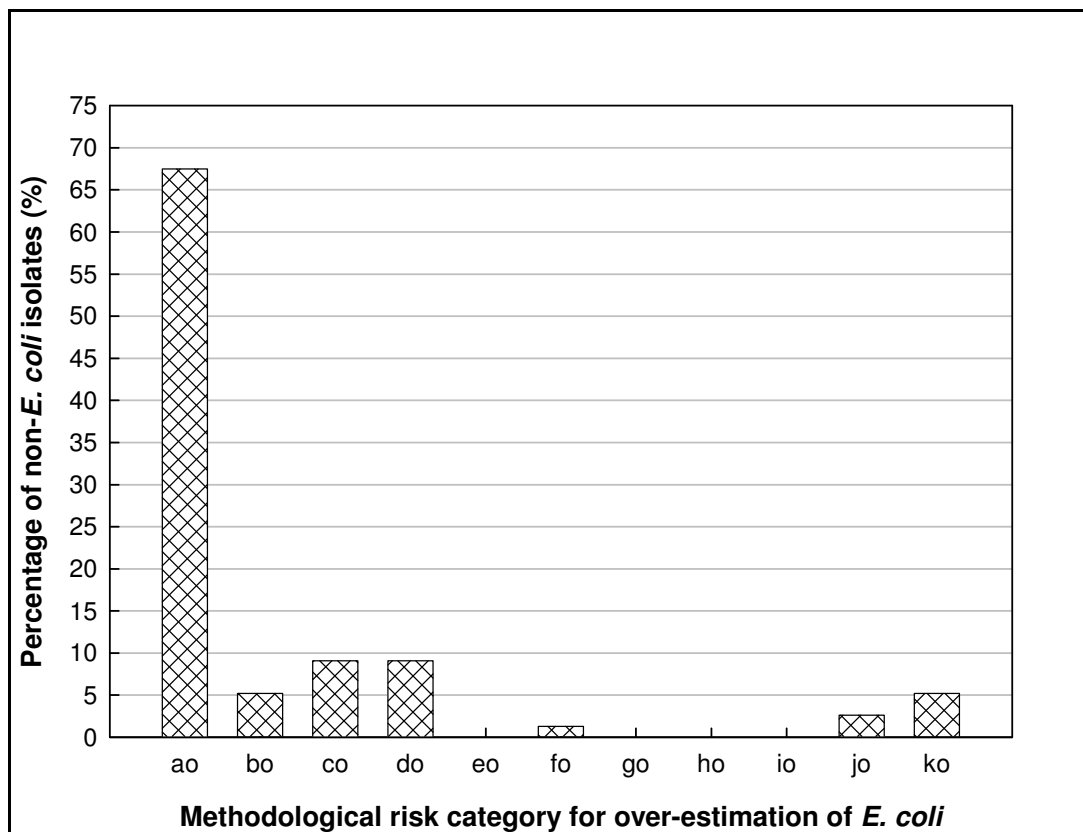
It is interesting to note that even without the confirmatory assurance of L-EMB agar, the distribution of isolates were predominantly in the higher end of the categories, indicating lower risk for the under-estimation of *E. coli*. In fact, the omission of L-EMB agar from the assay would result in the shift of nearly all isolates to the first three risk categories. The only isolates which remained at the high risk end of the histogram were those which were not detected by LST and/or BGLB broths. Therefore, the absence of L-EMB from the MTF assay increased the proportion of isolates in the first half of the categories from 85.3% to 88.2%, and reduced the number of isolates in the second half of the categories from 14.7% to 11.8%. When these results are seen in isolation, the immediate conclusion would be the removal of L-EMB agar from the assay to improve the accuracy of the enumeration of *E. coli*. However, these results do not account for the potential inaccuracies which could be caused by a failure to validate non-*E. coli* organisms which produced typical reactions in EC broth with MUG. A meaningful conclusion can only be made when this information is also considered carefully in conjunction with the results presented in Table 8.23 and Fig. 8.9.

Classification of non-*E. coli* isolates into categories for the risk of the over-estimation of *E. coli* by MTF included all isolates, coliform and non-coliform, which were not identified as *E. coli*. The results of this classification are shown in Table 8.20.

**Table 8.20** Individual isolates and their risk category for the over-estimation of *E. coli*

Isolate	Identity	Risk	Isolate	Identity	Risk	Isolate	Identity	Risk
2	<i>K. pneumoniae</i>	co	34	<i>Entb. asburiae</i>	ao	73-3	<i>Prot. mirabilis</i>	ko
3-2	<i>K. pneumoniae</i>	co	35	<i>S. marcescens</i>	ao	75	<i>R. ornithinolytica</i>	ao
4	<i>A. hydrophila</i>	ao	36	<i>Entb. asburiae</i>	ao	80	<i>Cit. freundii</i>	ao
5	<i>Entb. aerogenes</i>	ao	37	<i>B. pseudofirmus</i>	do	81-2	<i>K. pneumoniae</i>	co
8	<i>K. pneumoniae</i>	co	38	<i>K. oxytoca</i>	do	86	<i>R. ornithinolytica</i>	ao
9	<i>K. pneumoniae</i>	co	39	<i>H. alvei</i>	ao	87	<i>Entb. kobei</i>	ao
11-1	<i>Cit. braakii</i>	ko	40	<i>K. oxytoca</i>	do	88	<i>Entb. asburiae</i>	ao
11-2	<i>M. morgani</i>	ao	41	<i>Entb. cloacae</i>	ao	89	<i>S. marcescens</i>	ao
12	<i>Cit. braakii</i>	ao	42	<i>Entb. asburiae</i>	ao	90	<i>K. pneumoniae</i>	do
13-2	<i>Prov. alcalifaciens</i>	ko	43	<i>Entb. asburiae</i>	ao	92	<i>Cit. braakii</i>	ao
14	<i>Prov. alcalifaciens</i>	ao	44	<i>Prov. rettgeri</i>	ao	93	<i>Entb. kobei</i>	do
15	<i>K. pneumoniae</i>	ao	46	<i>K. pneumoniae</i>	ao	94	<i>Cit. freundii</i>	ao
17	<i>Entb. asburiae</i>	ao	47	<i>Cit. braakii</i>	ao	97	<i>Entb. asburiae</i>	bo
20	<i>K. pneumoniae</i>	jo	48-2	<i>S. marcescens</i>	ao	98	<i>Prov. stuartii</i>	co
21	<i>M. luteus</i>	ao	49	<i>K. pneumoniae</i>	do	99	<i>Prot. vulgaris</i>	ao
22	<i>M. morgani</i>	ao	50	<i>Entb. asburiae</i>	ao	100	<i>Prot. mirabilis</i>	ao
23	<i>M. morgani</i>	ko	51	<i>R. ornithinolytica</i>	jo	101	<i>Prot. vulgaris</i>	ao
24-1	<i>Entb. asburiae</i>	ao	53	<i>K. pneumoniae</i>	ao	102	<i>Prot. vulgaris</i>	ao
24-2	<i>M. morgani</i>	ao	56	<i>Entb. cloacae</i>	bo	103	<i>Prot. mirabilis</i>	ao
25	<i>Entb. radicincitans</i>	ao	57-2	<i>Entb. asburiae</i>	bo	104	<i>Prov. stuartii</i>	ao
26	<i>B. pseudofirmus</i>	ao	59	<i>K. pneumoniae</i>	ao	105	<i>Prot. mirabilis</i>	ao
27	<i>K. pneumoniae</i>	ao	64-2	<i>Cr. sakazakii</i>	fo	106	<i>Prot. mirabilis</i>	co
28	<i>K. pneumoniae</i>	ao	69	<i>Entb. asburiae</i>	ao	107	<i>Prot. vulgaris</i>	ao
29	<i>R. ornithinolytica</i>	do	70	<i>Entb. asburiae</i>	ao	108	<i>K. pneumoniae</i>	bo
30	<i>S. marcescens</i>	ao	71	<i>Entb. asburiae</i>	ao	109	<i>Prov. stuartii</i>	ao
32	<i>Entb. asburiae</i>	ao	72	<i>Entb. kobei</i>	ao			

The results in Table 8.20 indicate that a large number of non-*E. coli* isolates fell within the ao category, which is associated with no risk for the over-estimation of *E. coli*. This category described isolates which failed to produce a reaction in LST and/or BGLB broth, and the proportion of non-*E. coli* isolates which fell within this category was 67.5%. Therefore, the proportion of “no risk” organisms is higher for the over-estimation classification when compared to the under-estimation analysis, indicating that under-estimation may pose a greater problem for the enumeration of *E. coli* by MTF. In addition to this empirical evidence, this argument is also theoretically valid since error in a method is preferred to give conservative rather than optimistic results when risk assessments are performed. Once again, these results were visually depicted. The isolate distribution is given as Fig. 8.6.



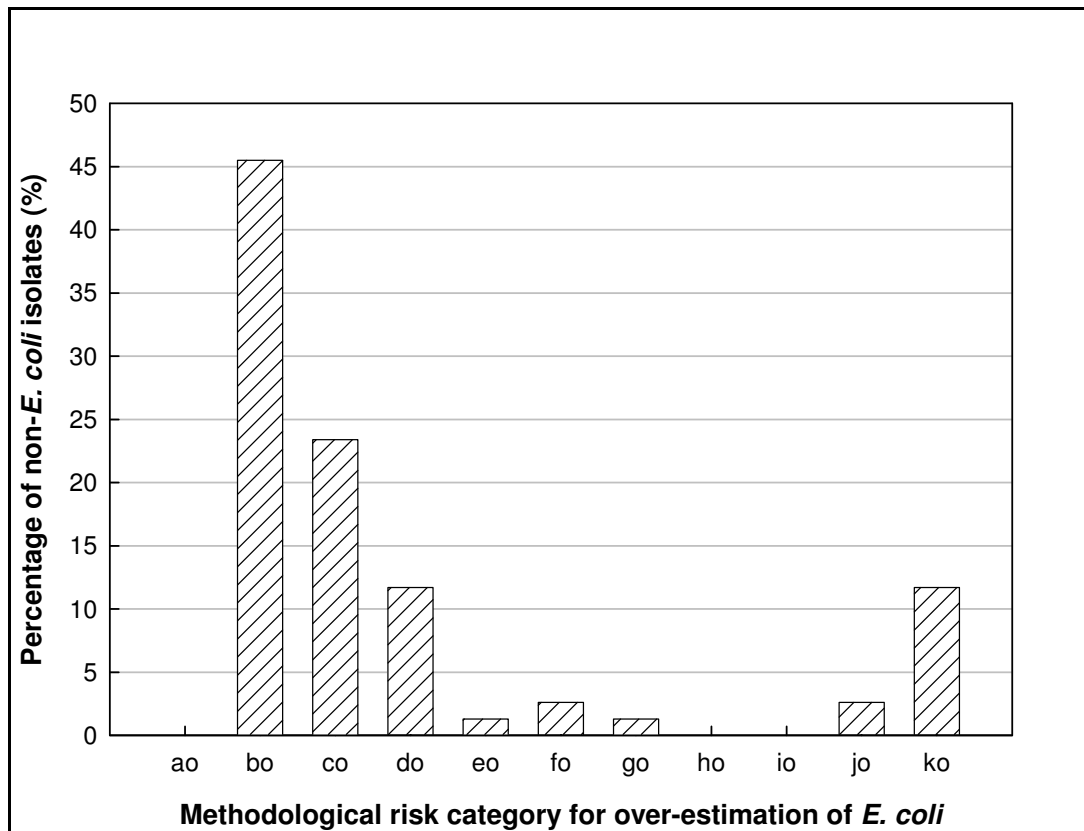
**Figure 8.6** Distribution of non-*E. coli* isolates (n=77) among risk categories for the over-estimation of *E. coli*

It is clear from the histogram presented in Fig. 8.6 that category ao comprised the majority of the isolates which were classified. Categories bo to fo described isolates with varying reactions in ascending degree of severity in EC broth with MUG, but all produced NMG colonies on L-EMB agar. Therefore, even if their presence in EC broth with MUG was erroneously interpreted as the presence of *E. coli*, as would be the case with category fo, their failure to produce metallic green colonies on L-EMB agar should correct the error in enumeration by EC broth with MUG. Of the remaining isolates, 19 fell into one of these categories.

Although the number of isolates which occurred on the high risk end of the histogram were considerably less than those which posed a lower risk for the over-estimation of *E. coli*, their reactions were extremely severe: two isolates (in category jo) produced growth and fluorescence in EC broth with MUG as well as metallic green colonies on L-EMB agar; and four isolates produced perfect typical reactions both in EC broth with MUG and on L-EMB agar. The latter group of isolates belonged to the genera *Citrobacter* (11-1), *Morganella* (23), *Providencia* (13-2) and *Proteus* (73-3).

Due to the observation in earlier chapters, as well as the known bacterial heterogeneity of the sample when it is inoculated into LST broth, the likelihood of organisms assigned to category

ao co-occurring with aerogenic organisms is great. For this reason, the category ao criterion was removed from the risk classification to determine the potential contribution of organisms in this category to the risk of over-estimation of *E. coli*, since the purpose of the evaluation was to determine the maximum possible *potential* of an organism to decrease the accuracy of enumeration done by MTF. The results of the revised evaluation are presented in Fig. 8.7.



**Figure 8.7** Distribution of non-*E. coli* isolates (n=77) among risk categories for the over-estimation of *E. coli*, excluding category ao from the risk categorisation

It is clear from Fig. 8.7 that a large proportion of isolates which previously resorted under category ao were reclassified to category bo when the former category was removed, to result in a total number of 35 isolates in category bo. This category describes isolates which could not produce any reaction in EC broth with MUG, but grew as NMG colonies on L-EMB agar when streaked out. It is clear from the description that the likelihood of these organisms being counted as *E. coli* is exceedingly slim.

The reclassification as a whole resulted in an increase of 40 isolates in categories bo to fo, which pose a low risk for over-estimation. However, 12 isolates which previously occurred in category ao were reclassified to higher methodological risk categories. One of the aforementioned isolates was reclassified to category go, which describes no reaction in EC broth with MUG but metallic green colonies on L-EMB agar. The likelihood of such an organism being enumerated is

not great, unless streaking on L-EMB is done. However, the increase of five isolates in category ko is troubling, since these maximum risk isolates now makes 11.7% of the total isolates classified. Even so, when the proportions across the histogram are calculated it is found that 84.4% of isolates occurred in the first half of the categories, while 15.6% of isolates occurred in the second half of the categories. This corresponds well with 85.3% of *E. coli* isolates occurring in the first half of the categories for the risk of under-estimation of *E. coli*.

The minimum concentrations of all problem category non-*E. coli* isolates in the original sample, calculated from the serial dilution from which they were isolated, are given in Table 8.21. These concentrations indicate the minimum number of these problematic organisms in the sample.

**Table 8.21** Minimum concentrations of isolates assigned to risk categories for the over-estimation of *E. coli*

Isolate	Minimum concentration range (cfu.mL <sup>-1</sup> )	Risk	Isolate	Minimum concentration range (cfu.mL <sup>-1</sup> )	Risk	Isolate	Minimum concentration range (cfu.mL <sup>-1</sup> )	Risk
2	10 <sup>0</sup>	co	37	10 <sup>0</sup>	do	73-3	10 <sup>0</sup>	ko
3-2	10 <sup>1</sup>	co	38	10 <sup>1</sup>	do	81-2	10 <sup>0</sup>	co
8	10 <sup>1</sup>	co	40	10 <sup>0</sup>	do	90	10 <sup>1</sup>	do
9	10 <sup>1</sup>	co	49	10 <sup>0</sup>	do	93	10 <sup>0</sup>	do
11-1	10 <sup>0</sup>	ko	51	10 <sup>0</sup>	jo	97	10 <sup>1</sup>	bo
13-2	10 <sup>1</sup>	ko	56	10 <sup>1</sup>	bo	98	10 <sup>0</sup>	co
20	10 <sup>3</sup>	jo	57-2	10 <sup>0</sup>	bo	106	10 <sup>0</sup>	co
23	10 <sup>0</sup>	ko	64-2	10 <sup>0</sup>	fo	108	10 <sup>1</sup>	bo
29	10 <sup>0</sup>	do						

The concentrations in Table 8.21 show a range of 10<sup>0</sup> to 10<sup>3</sup> cfu.mL<sup>-1</sup> for the problematic non-*E. coli* isolates. From these concentrations it once again appears that risk category alone does not predict the potential of an isolate to cause inaccuracies, but that the concentration of the organism in the original sample should be considered. When these concentrations are used to transform the qualitative information of the risk classification to quantitative information through the use of concentrations, the true values should be determined since the use of minimum concentrations cannot be justified. Despite this, and as mentioned previously, the use of minimum concentrations here serve as an illustration of the quantitative approach which could be used in a more refined version of this risk classification.

As was done for the risk classification relating to the under-estimation of *E. coli*, the assay was examined when fluorescence was omitted as a criterion to determine the influence of MUG. Differences were expected due to the presence of  $\beta$ -D-glucuronidase negative strains which could produce gas and growth in EC broth with MUG. If fluorescence was to be omitted from the criteria, these isolates should be classified with aerogenic  $\beta$ -D-glucuronidase positive isolates in this

evaluation. Since no great reduction in under-estimation could be observed with the omission of MUG, the results reported in this section will be of interest for making conclusions relating to the necessity of MUG in the assay. The results of the modified risk classification are presented in Table 8.22. For the reason explained previously, non-detection by LST and/or BGLB was removed as a criterion.

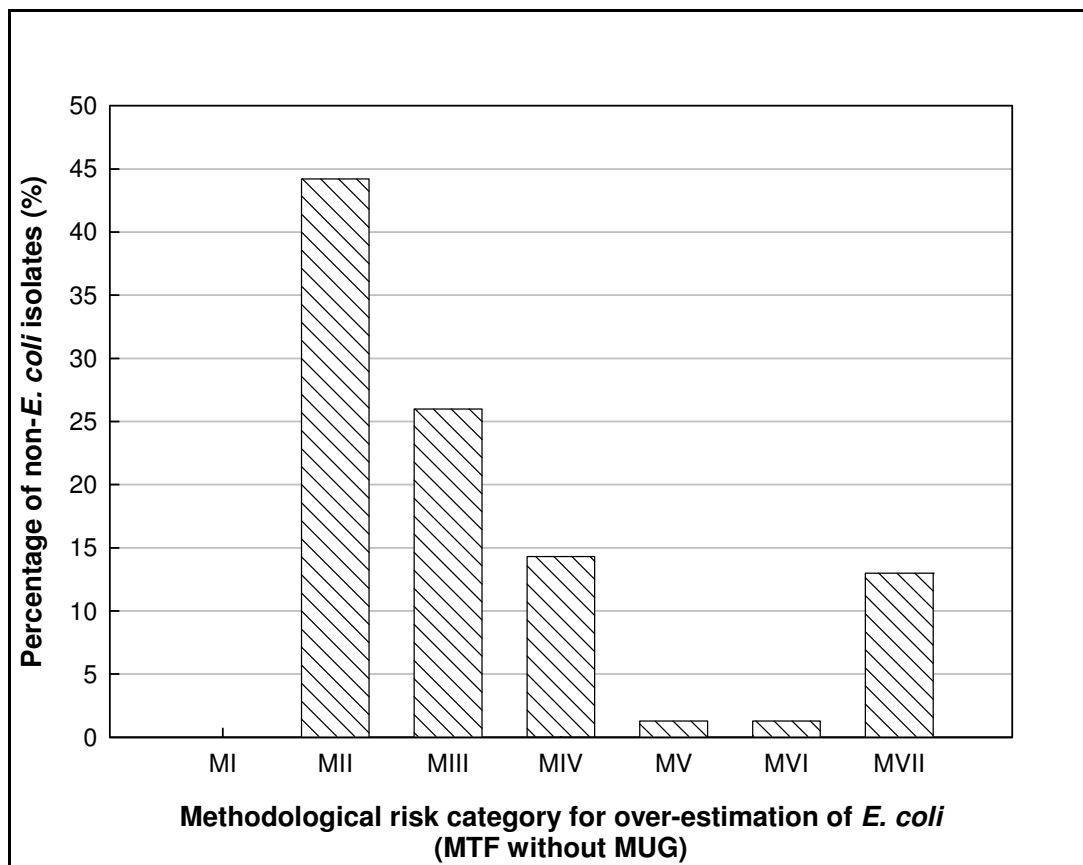
**Table 8.22** Individual isolates and their risk category for the over-estimation of *E. coli* in an MTF assay with no MUG

Isolate	Identity	Risk	Isolate	Identity	Risk	Isolate	Identity	Risk
2	<i>K. pneumoniae</i>	MIII	34	<i>Entb. asburiae</i>	MVII	73-3	<i>Prot. mirabilis</i>	MVII
3-2	<i>K. pneumoniae</i>	MIII	35	<i>S. marcescens</i>	MII	75	<i>R. ornithinolytica</i>	MII
4	<i>A. hydrophila</i>	MII	36	<i>Entb. asburiae</i>	MIII	80	<i>Cit. freundii</i>	MVII
5	<i>Entb. aerogenes</i>	MII	37	<i>B. pseudofirmus</i>	MIV	81-2	<i>K. pneumoniae</i>	MIII
8	<i>K. pneumoniae</i>	MIII	38	<i>K. oxytoca</i>	MIV	86	<i>R. ornithinolytica</i>	MVII
9	<i>K. pneumoniae</i>	MIII	39	<i>H. alvei</i>	MII	87	<i>Entb. kobei</i>	MII
11-1	<i>Cit. braakii</i>	MVII	40	<i>K. oxytoca</i>	MIV	88	<i>Entb. asburiae</i>	MII
11-2	<i>M. morgani</i>	MVII	41	<i>Entb. cloacae</i>	MIV	89	<i>S. marcescens</i>	MII
12	<i>Cit. braakii</i>	MV	42	<i>Entb. asburiae</i>	MIV	90	<i>K. pneumoniae</i>	MIV
13-2	<i>Prov. alcalifaciens</i>	MVII	43	<i>Entb. asburiae</i>	MIII	92	<i>Cit. braakii</i>	MII
14	<i>Prov. alcalifaciens</i>	MII	44	<i>Prov. rettgeri</i>	MII	93	<i>Entb. kobei</i>	MIV
15	<i>K. pneumoniae</i>	MII	46	<i>K. pneumoniae</i>	MIII	94	<i>Cit. freundii</i>	MII
17	<i>Entb. asburiae</i>	MII	47	<i>Cit. braakii</i>	MIII	97	<i>Entb. asburiae</i>	MII
20	<i>K. pneumoniae</i>	MVII	48-2	<i>S. marcescens</i>	MIV	98	<i>Prov. stuartii</i>	MIII
21	<i>M. luteus</i>	MII	49	<i>K. pneumoniae</i>	MIV	99	<i>Prot. vulgaris</i>	MII
22	<i>M. morgani</i>	MII	50	<i>Entb. asburiae</i>	MIII	100	<i>Prot. mirabilis</i>	MIII
23	<i>M. morgani</i>	MVII	51	<i>R. ornithinolytica</i>	MVI	101	<i>Prot. vulgaris</i>	MII
24-1	<i>Entb. asburiae</i>	MII	53	<i>K. pneumoniae</i>	MVII	102	<i>Prot. vulgaris</i>	MII
24-2	<i>M. morgani</i>	MII	56	<i>Entb. cloacae</i>	MII	103	<i>Prot. mirabilis</i>	MIII
25	<i>Entb. radicincitans</i>	MII	57-2	<i>Entb. asburiae</i>	MII	104	<i>Prov. stuartii</i>	MIII
26	<i>B. pseudofirmus</i>	MII	59	<i>K. pneumoniae</i>	MII	105	<i>Prot. mirabilis</i>	MIII
27	<i>K. pneumoniae</i>	MIII	64-2	<i>Cr. sakazakii</i>	MIV	106	<i>Prot. mirabilis</i>	MIII
28	<i>K. pneumoniae</i>	MII	69	<i>Entb. asburiae</i>	MII	107	<i>Prot. vulgaris</i>	MII
29	<i>R. ornithinolytica</i>	MIV	70	<i>Entb. asburiae</i>	MII	108	<i>K. pneumoniae</i>	MIII
30	<i>S. marcescens</i>	MII	71	<i>Entb. asburiae</i>	MII	109	<i>Prov. stuartii</i>	MIII
32	<i>Entb. asburiae</i>	MIII	72	<i>Entb. kobei</i>	MII			

When MUG is excluded from the MTF assay through the omission of the fluorescence criterion in EC broth with MUG, the number of isolates occurring in the first category (MII, due to the exclusion of LST and BGLB non-detection) is 34. This is a reduction of one isolate when compared to the number of isolates in the first category for an assay which did include MUG. The isolate which did not correspond to the first “no risk” category for this classification was isolate 12, which was identified as *Citrobacter braakii*. This organism did not fall into the MII category due to its production of metallic green colonies on L-EMB agar, in contrast with the NMG colonies



produced by the other isolates which were previously categorised into category bo. Therefore, the omission of MUG did not cause a considerable change in the amount of isolates which pose virtually no risk for over-estimation. No judgment can be made on the necessity of MUG until the distribution of isolates among the other categories has been shown. This distribution is depicted in Fig. 8.8.



**Figure 8.8** Distribution of non-*E. coli* isolates (n=77) among risk categories for the over-estimation of *E. coli* in an MTF assay without MUG, excluding category MI from the risk categorisation

It is evident from Fig. 8.8 that most of the isolates were still grouped on the low risk end of the histogram, with 84.4% occurring in the first half of the categories. In contrast, 15.6% of the isolates were situated in the high risk half of the histogram. These proportions are identical to the proportions obtained during the risk classification which did included fluorescence as a criterion.

The amount of maximum risk isolates increased from nine in an assay with MUG to ten in one without, where category MVII described growth and gas in EC broth with MUG accompanied by a subsequent production of metallic green colonies on L-EMB agar. The organism which was reclassified to the maximum risk category was isolate 20, *Klebsiella pneumoniae*. When fluorescence was removed as a criterion for maximum risk this organism elicited all the reactions required for the new maximum risk category, namely gas and growth in EC broth and metallic

green colonies on L-EMB agar. However, the contribution, percentage wise, of one isolate amounts to 1.3%, which is an extremely small increase in the amount of maximum risk isolates. In addition, molecular identification of this isolate with *uidA*, *tuf* and *mdh* genes showed that it is *E. coli*. Furthermore, the same percentage (1.3%) contributes to the decrease in no risk isolates from 35 to 34.

Therefore, in combination with the results reported for the omission of MUG from the MTF assay and its effect on the under-estimation of *E. coli*, it can be provisionally concluded that the presence of MUG in EC broth did not appreciably decrease the risk of under- or over-estimation of *E. coli* by MTF. Furthermore, the observation of fluorescence in EC broth with MUG can be extremely subjective, which could result in the presence of MUG causing a larger degree of misinterpretation with no concurrent decrease in the risk of under- or over-estimation of *E. coli*.

The influence of the inclusion of L-EMB agar as confirmatory step in the MTF method was also evaluated by omitting growth on L-EMB agar as a criterion. It was shown in the previous section that the absence of L-EMB agar increased the number of *E. coli* isolates in the low risk half of categories for the under-estimation of *E. coli* by approximately 3.0%. The results of the risk classification done without L-EMB agar data is shown in Table 8.23. Once again, non-detection by LST and/or BGLB was removed as a criterion to assess the maximum potential of the isolates to cause over-estimation.

**Table 8.23** Individual isolates and their risk category for the over-estimation of *E. coli* in an MTF assay with no confirmation by L-EMB agar

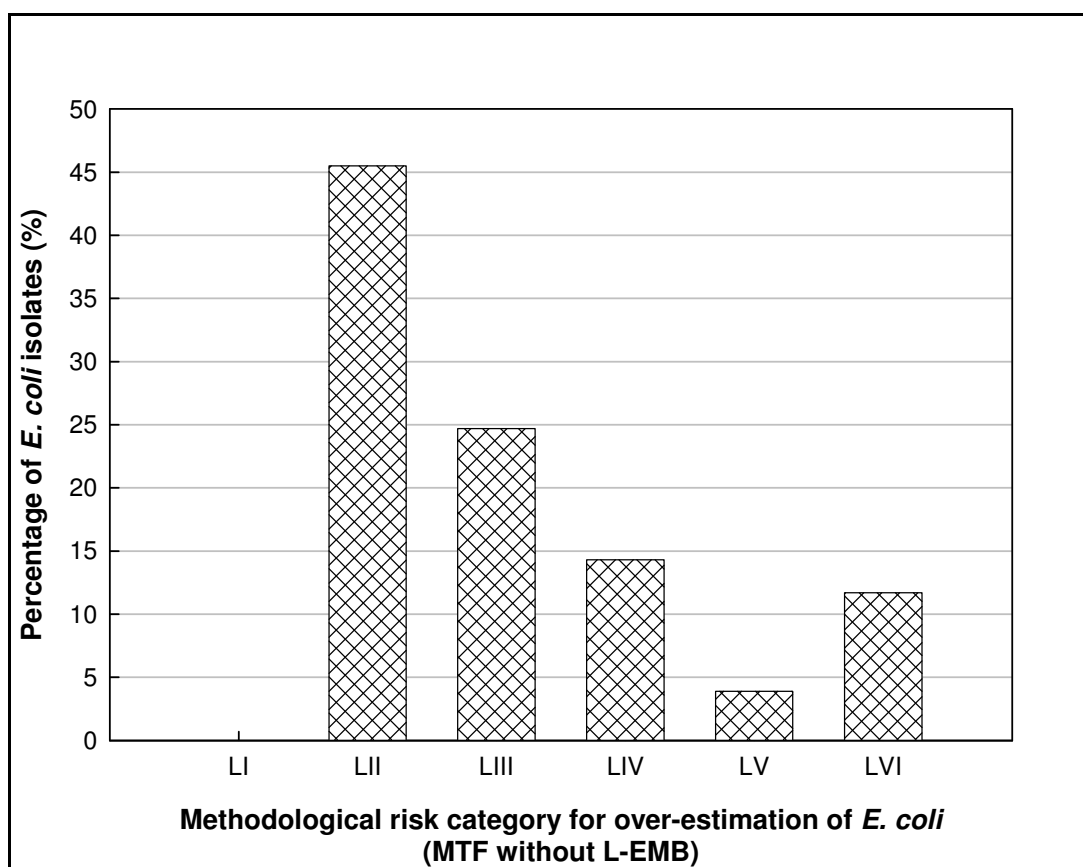
Isolate	Identity	Risk	Isolate	Identity	Risk	Isolate	Identity	Risk
2	<i>K. pneumoniae</i>	LIII	34	<i>Entb. asburiae</i>	LI	73-3	<i>Prot. mirabilis</i>	LVI
3-2	<i>K. pneumoniae</i>	LIII	35	<i>S. marcescens</i>	LI	75	<i>R. ornithinolytica</i>	LI
4	<i>A. hydrophila</i>	LI	36	<i>Entb. asburiae</i>	LI	80	<i>Cit. freundii</i>	LI
5	<i>Entb. aerogenes</i>	LI	37	<i>B. pseudofirmus</i>	LIV	81-2	<i>K. pneumoniae</i>	LIII
8	<i>K. pneumoniae</i>	LIII	38	<i>K. oxytoca</i>	LIV	86	<i>R. ornithinolytica</i>	LI
9	<i>K. pneumoniae</i>	LIII	39	<i>H. alvei</i>	LI	87	<i>Entb. kobei</i>	LI
11-1	<i>Cit. braakii</i>	LVI	40	<i>K. oxytoca</i>	LIV	88	<i>Entb. asburiae</i>	LI
11-2	<i>M. morganii</i>	LI	41	<i>Entb. cloacae</i>	LI	89	<i>S. marcescens</i>	LI
12	<i>Cit. braakii</i>	LI	42	<i>Entb. asburiae</i>	LI	90	<i>K. pneumoniae</i>	LIV
13-2	<i>Prov. alcalifaciens</i>	LVI	43	<i>Entb. asburiae</i>	LI	92	<i>Cit. braakii</i>	LI
14	<i>Prov. alcalifaciens</i>	LI	44	<i>Prov. rettgeri</i>	LI	93	<i>Entb. kobei</i>	LIV
15	<i>K. pneumoniae</i>	LI	46	<i>K. pneumoniae</i>	LI	94	<i>Cit. freundii</i>	LI
17	<i>Entb. asburiae</i>	LI	47	<i>Cit. braakii</i>	LI	97	<i>Entb. asburiae</i>	LII
20	<i>K. pneumoniae</i>	LIV	48-2	<i>S. marcescens</i>	LI	98	<i>Prov. stuartii</i>	LIII
21	<i>M. luteus</i>	LI	49	<i>K. pneumoniae</i>	LIV	99	<i>Prot. vulgaris</i>	LI
22	<i>M. morganii</i>	LI	50	<i>Entb. asburiae</i>	LI	100	<i>Prot. mirabilis</i>	LI
23	<i>M. morganii</i>	LIV	51	<i>R. ornithinolytica</i>	LV	101	<i>Prot. vulgaris</i>	LI
24-1	<i>Entb. asburiae</i>	LI	53	<i>K. pneumoniae</i>	LI	102	<i>Prot. vulgaris</i>	LI
24-2	<i>M. morganii</i>	LI	56	<i>Entb. cloacae</i>	LII	103	<i>Prot. mirabilis</i>	LI

**Table 8.23 continued**

Table 8.23 continued

Isolate	Identity	Risk	Isolate	Identity	Risk	Isolate	Identity	Risk
25	<i>Entb. radicincitans</i>	LI	57-2	<i>Entb. asburiae</i>	LII	104	<i>Prov. stuartii</i>	LI
26	<i>B. pseudofirmus</i>	LI	59	<i>K. pneumoniae</i>	LI	105	<i>Prot. mirabilis</i>	LI
27	<i>K. pneumoniae</i>	LI	64-2	<i>Cr. sakazakii</i>	LVI	106	<i>Prot. mirabilis</i>	LIII
28	<i>K. pneumoniae</i>	LI	69	<i>Entb. asburiae</i>	LI	107	<i>Prot. vulgaris</i>	LI
29	<i>R. ornithinolytica</i>	LIV	70	<i>Entb. asburiae</i>	LI	108	<i>K. pneumoniae</i>	LIII
30	<i>S. marcescens</i>	LI	71	<i>Entb. asburiae</i>	LI	109	<i>Prov. stuartii</i>	LI
32	<i>Entb. asburiae</i>	LI	72	<i>Entb. kobei</i>	LI			

The results of the risk classification revealed that 35 isolates occurred in the first category (LII, due to the exclusion of LST and BGLB non-detection) when L-EMB agar confirmation was omitted from the MTF assay. The same number of isolates occurred in the first category when the risk classification was done with the inclusion of L-EMB agar in the MTF (see Fig. 8.7). The distribution of isolates among the rest of the risk categories are shown in the histogram presented as Fig. 8.9.



**Figure 8.9** Distribution of non-*E. coli* isolates (n=77) among risk categories for the over-estimation of *E. coli* in an MTF assay without L-EMB confirmation, excluding category LI from the risk categorisation

As mentioned previously, 35 isolates occurred in the first category (LII) when the risk classification was done on the MTF assay without L-EMB agar. This category describes a failure of isolates to produce gas, growth or fluorescence in EC broth with MUG. Since the number of isolates in this category remained constant irrespective of the inclusion of L-EMB agar, it can be concluded that all isolates which failed to produce any reaction in EC broth with MUG grew either as NMG colonies, or did not grow at all, on L-EMB agar. When examining the results presented in Tables 8.9 to 8.12, it was found that 34 of these isolates failed to produce any growth on L-EMB agar, and only isolate 99 was capable of producing light purple colonies on L-EMB agar despite its failure to elicit any reaction in EC broth with MUG. Therefore, it can be concluded that growth on L-EMB agar by organisms which did not produce any reaction in EC broth with MUG is not an important consideration for the method.

Nine isolates were categorised into category LVI, which describes a perfect typical reactions in EC broth with MUG. These isolates were previously classified in Fig. 8.7 as belonging to category ko, which described the production of gas, growth and fluorescence in EC broth with MUG and subsequent production of metallic green colonies on L-EMB agar. Therefore, the number of isolates in the maximum risk category remained unaffected by the exclusion of L-EMB agar from the assay.

However, when the distribution of isolates in Fig. 8.9 are used to calculate the proportion of isolates occurring in the low risk end of the histogram, it was found that 77.3% were found in the first half of the risk categories. This proportion shows a decrease in 7.1% from the 84.4% of isolates occurring in the low risk end of the histogram for an assay which included L-EMB agar. The exclusion of L-EMB agar from the MTF assay resulted in a decrease of 3.0% in high risk *E. coli* isolates during the risk classification for the under-estimation of *E. coli* (see Fig. 8.5). When weighing up the exclusion of L-EMB agar from the assay, the beneficial impact on the under-estimation as well as the detrimental impact on the over-estimation of *E. coli* should be considered. With the isolates at our disposal, the results in Fig. 8.7 and 8.9 show that the detrimental effect (7.1% increase in high risk for over-estimation isolates) surpass the beneficial outcome (3.0% decrease in high risk for under-estimation isolates) of omitting L-EMB agar from the MTF assay. It should be kept in mind, however, that for the under-estimation of *E. coli* the amount of first category (no risk) isolates increased by 5.9% (see Fig. 8.3 and 8.5) when L-EMB agar was omitted.

#### Summation of methodological risk classification for the under- and over-estimation of coliforms

A summary of the results obtained from the risk classifications for the under- and over-estimation of coliforms is given in Table 8.24. The results reflected in the table indicates the percentage of isolates in the first category, which represents no risk, and the percentages of isolates in low risk and high risk halves of the risk classification histogram. Finally, the table also indicates the

percentage of isolates which occurred in the last category, which corresponds to the maximum risk for under- or over-estimation.

It is evident from Table 8.24 on the next page that the risk of under-estimation of coliforms by MTF can be considerable, with 35.0% of isolates occurring in the high risk half of the risk categories. This is attributable to a large proportion of coliforms with varying profiles of anaerogenicity in LST and BGLB broths.

**Table 8.24** Summary of results from risk classifications for the under- and over-estimation of coliforms<sup>‡</sup>

<b>Risk classification</b>	<b>Isolates in first category (%)</b>	<b>Isolates in low risk half (%)</b>	<b>Isolates in high risk half (%)</b>	<b>Isolates in last category (%)</b>
Under-estimation of coliforms (n=107)	50.5	65.0	35.0	3.7
Over-estimation of coliforms (n=4)	25.0	50.0	50.0	25.0

<sup>‡</sup>Percentages calculated from the total number of isolates classified

However, it should be noted that a very small proportion, only 3.7%, of the isolates occurred in the maximum risk category, i.e. failed to grow or produce gas in LST broth. In addition, 50.5% of isolates occurred in the first category, which describes isolates which pose no risk for under-estimation of coliforms. Therefore, according to the results of these isolates, the risk of under-estimating coliform numbers to any degree is 49.5%, but the percentage of isolates which would cause a high risk for under-estimation is 35.0%, with a guaranteed 3.7% under-estimation.

Results obtained from the risk classification for the over-estimation of coliforms should be interpreted cautiously, due to the extremely small sample set which was used. Of the 111 isolates which were obtained from both atypical and typical reactions over all steps of the MTF method, only four non-coliforms were found. This in itself should be telling regarding the number of non-coliforms which can grow in media of the MTF. The composition of the media is specifically designed to inhibit the growth of non-coliform organisms and, therefore, it is unsurprising that only four non-coliforms were isolated. One isolate did, however, grow and produce gas in both LST and BGLB broths, and was classified within the maximum risk category. This isolate (37) was identified as *Bacillus pseudofirmus*. Further analyses on this organism may reveal that it is in co-habitation with a coliform, or it may be an extremely anomalous strain of *B. pseudofirmus*.

Summation of methodological risk classification for the under- and over-estimation of *E. coli*

A summary of the results of the methodological risk classification for the under- and over-estimation of *E. coli* is given as Table 8.25. These results are reported as the percentages of isolates in the first “no-risk” category, isolates in low risk and high risk halves of the risk

classification histogram, and isolates which were assigned to the maximum risk category in each case.

**Table 8.25** Summary of results from risk classifications for the under- and over-estimation of *E. coli*<sup>‡</sup>

Risk classification	Isolates in first category (%)	Isolates in low risk half (%)	Isolates in high risk half (%)	Isolates in last category (%)
Under-estimation of <i>E. coli</i> (n=34)	50.0	85.3	14.7	11.8
Under-estimation of <i>E. coli</i> without MUG (n=34)	70.6	85.3	14.7	11.8
Under-estimation of <i>E. coli</i> without L-EMB agar (n=34)	55.9	88.2	11.8	11.8
Over-estimation of <i>E. coli</i> (n=77)	45.5	84.4	15.6	11.7
Over-estimation of <i>E. coli</i> without MUG (n=77)	44.2	84.4	15.6	13.0
Over-estimation of <i>E. coli</i> without L-EMB agar (n=77)	45.5	77.3	22.7	11.7

<sup>‡</sup>Percentages calculated from the total number of isolates classified

At first glance, the risk assessments for the both the under- and over-estimation of *E. coli*, seen in Table 8.25, are far more favourable than that of the under-estimation of coliforms. This is probably attributable in part to the higher homogeneity in bacterial species in EC broth with MUG and on L-EMB agar, when compared to LST and BGLB broths. However, for the under-estimation of *E. coli*, 50.0% of isolates posed no risk while 11.8% posed the maximum risk of under-estimation. Both these proportions are less favourable than their counterparts for the under-estimation of coliforms. Despite the fact that the risk of under-estimating *E. coli* by any degree is 50.0%, only 14.7% of isolates posed a high risk of being under-estimated.

The over-estimation of *E. coli* also contributes to the inaccuracy of the method, with only 45.5% of isolates occurring in the category which poses no risk for over-estimation. Similar to its corresponding category for under-estimation, the maximum risk category for over-estimation described 11.7% of the isolates. These numbers, although unfavourable for the accuracy of the method, is preferable on the side of over-estimation rather than under-estimation. This is because the MTF method is frequently used to enumerate coliforms and *E. coli* as part of risk assessments done on both water and food.

Since the thermotolerant coliforms and *E. coli* are so closely related, it is extremely difficult to suggest a measure which will exclude the former while including all within the latter group. The most valuable compound known currently is MUG, used to test for the presence of  $\beta$ -D-

glucuronidase in *E. coli*, which is already included within the MTF protocol and contributes its own fallacies to the method. For example, it has been shown by the results in this chapter and by literature that several *E. coli* strains cannot hydrolyse MUG (Chang *et al.*, 1989; Manafi, 2000; Pisciotta *et al.*, 2002; Rompré *et al.*, 2002), while many non-*E. coli* coliforms are capable of hydrolysing MUG (Chang *et al.*, 1989; Geissler *et al.*, 2000; Manafi, 2000; Pisciotta *et al.*, 2002; Fricker *et al.*, 2010), which would result in both beneficial and detrimental impacts on the method.

## 8.5 CONCLUSIONS

Before any concluding remarks are made regarding the results which have been presented, it should be acknowledged that the collection of isolates which were used to generate these results were obtained from a single water type which had been sourced over a period of 18 months from rivers within close geographical proximity to each other. Therefore, the conclusions drawn regarding the accuracy of the method and the determined influences of MUG and L-EMB agar to the MTF assay are based on a distinct group of isolates, and cannot necessarily be extended to any water type or water obtained from any location.

Several important conclusions arose from the results reported within this chapter. Some of these conclusions impact negatively on the MTF method, while some were positive. The first of these implications is the negative influence of anaerogenic coliforms on the accuracy of the enumeration procedure. In addition, synergistic reactions between organisms, the existence of organisms which react perfectly as false positives for *E. coli*, as well as transference of non-target organisms during the method were demonstrated empirically or postulated based on the findings reported here. However, when an analysis of the method was done using a risk classification approach, it was found that the under- and over-estimation of coliforms, but especially *E. coli*, was not dramatically affected by most of the aforementioned complications. Evaluating the contribution of MUG and L-EMB agar in the MTF assay revealed that the omission of L-EMB agar and, especially, MUG would increase the accuracy and have no appreciable effect on the method, respectively.

### 8.5.1 Isolation and transference of non-target organisms

The isolation technique took the form of a “grab sample” with an inoculation loop, and the reactions in the tube were attributed to the organism which was isolated from it. This reasoning was flawed in some cases, particularly in the first two broths steps where the bacterial population is still fairly heterogeneous. This was shown to be the case through the observation in the results of the individual MTF experiment that many isolates, especially in LST and BGLB broths, which were obtained from typical reactions, could not reproduce all or parts of the typical reaction. Therefore,



it was concluded that another organism or organisms were responsible for the typical reaction or the complementary parts of the typical reaction.

Although this phenomenon, which is intrinsically part of the isolation procedure, is problematic for the purposes of studying the bacteria responsible for atypical reactions throughout the MTF method, it is far more concerning for the method itself. Since the transference of organisms from a positive medium is done by transferring an inoculation loop full of this medium to the next step in the MTF protocol, the same inadvertent transference of non-target organisms could occur. Worse yet, this random sampling of the contents of the medium could result in the intended test organisms being “missed”, which could result in an undeterminable number of under-estimations.

### **8.5.2 Suppression of true positives resulting in false negatives**

During the individual MTF experiment it was found that isolates from LST and BGLB broths, which were found to be anaerogenic during river water MTF analyses, could produce gas when inoculated individually. These isolates comprised 54.5% and 66.7% of originally anaerogenic isolates from LST and BGLB broths, respectively. This phenomenon may be explained through the higher starting inoculum used during the individual MTF analyses, which would have increased the likelihood of gas production both through increased isolate numbers and the absence of competing bacteria. It is recommended that a more in-depth quantitative evaluation of this phenomenon be conducted to determine the influence of isolate concentration in a bacterially heterogeneous sample on MTF accuracy.

### **8.5.3 Synergistic and true false positives**

The observation that some organisms which were initially isolated from typical reactions could not reproduce the typical reactions in the same media when they were inoculated individually raised an important secondary implication: the existence of synergistic false positives. This phenomenon was shown in many instances during the individual MTF experiment, where the complement of the initial typical reaction was produced by more than one organism, each with their own complete or incomplete typical reaction. For instance, isolates 64-1 and 64-2 (see Table 7.11) were isolated from a tube of EC broth with MUG which exhibited gas, growth and fluorescence. When the isolates were tested individually, however, isolate 64-2 was found only to produce growth and fluorescence. Fortunately, the complementary organism (64-1) was *E. coli*, and the enumeration would have remained unaffected. If, however, isolate 64-2 co-occurred with isolate 90 (see Table 7.11), the gas production caused by the latter would also have resulted in a perfect typical reaction in EC broth with MUG by two non-*E. coli* organisms.

In addition to the false positive reactions caused by synergy between organisms, a number of true false positives were also identified. These isolates were able to grow and produce gas and fluorescence in EC broth with MUG, as well as grow as metallic green colonies on L-EMB agar.

Within the group of 77 non-*E. coli* isolates, nine organisms (11.7%) were able to produce this false positive reaction. Five of these were admittedly anaerogenic in either LST or BGLB broth (or both), but the phenomenon of synergistic reactions in the media could readily cause these organisms to be inadvertently transferred to EC broth with MUG.

#### **8.5.4 Under- and over-estimation of coliforms**

The results of the methodological risk classification relating to the under-estimation of coliforms (see Table 8.24) showed that more than half of isolates tested did not pose any methodological risk. However, under-estimation can be problematic for the MTF method, with 35.0% of isolates assigned to high risk categories for under-estimation. This proportion of isolates is primarily attributable to an inability to produce gas from lactose in MTF media. Therefore, anaerogenic coliforms are the primary contributors to the under-estimation of coliforms by MTF and the degree of under-estimation will be a function of the proportion of anaerogenic coliforms in the sample. Ironically, the synergistic false positive reactions which were described in the previous section, or co-occurrence with an aerogenic coliform, will allow many of these high risk coliforms to pass normally through the steps of the MTF method and thereby reduce the percentage of isolates in high risk categories.

The assigning of 35.0% of isolates in high risk categories still remains troubling, since this method is frequently used in microbiological risk assessments of both foodstuffs and water. It is recommended that some measure, such as the inclusion of a  $\beta$ -D-galactosidase specific substrate, be incorporated within the first MTF medium to assist in the detection of anaerogenic coliforms.

The results for the analysis of methodological risk of coliform over-estimation were largely inconclusive due to the small isolate set. This limited set of isolates in itself is indicative of the small risk posed by non-coliforms. Since the isolation procedure targeted MTF reactions which were either incomplete or discrepant from the normal positive reactions, the expectation would be to find numerous non-target organisms. This was, however, not the case and only four non-coliforms were identified from 111 isolates. Based on these observations, the conclusion regarding the over-estimation of coliforms by MTF is that this type of enumeration inaccuracy is rarely encountered due to the chemical inhibition of non-coliforms. Furthermore, the presence of a false positive coliform is most likely inconsequential to the method accuracy due to the high probability of co-occurrence with true coliforms.

#### **8.5.5 Under- and over-estimation of *E. coli***

The results of the methodological risk classification relating to inaccuracies in the enumeration of *E. coli* (see Table 8.25) showed that both under- and over-estimation occurred, and that these inaccuracies impacted nearly equally on the method. The results are encouraging for the reliability of *E. coli* results generated by the method, since high percentages of isolates were assigned to no risk categories for both the under-estimation (50.0%) and over-estimation (45.5%). Furthermore,

low percentages of isolates were assigned to maximum risk categories for under-estimation (11.8%) and over-estimation (11.7%). While these results show that MTF is reasonably efficient at detecting only target organisms, the presence of isolates which pose methodological risk for the under-estimation of *E. coli* is troublesome since this inaccuracy could lead to false complacency. The over-estimation of *E. coli* is, from a methodological viewpoint, equally troublesome; but is preferable to under-estimation since it will result in a more conservative enumeration. Since the results generated with this method are used to determine the safety of food and water for human contact or consumption, an error on the side of caution is more acceptable than the alternative.

#### The influence of MUG on the accuracy of the MTF method

The results in Table 8.25 show that the exclusion of MUG from the assay resulted in a considerable increase in isolates which did not pose any risk for under-estimation to 70.6%, which decreased the possibility of any degree of under-estimation to 29.4%. Additionally, this omission of MUG from the assay did not increase the proportion of high or maximum risk isolates. For the over-estimation of *E. coli*, the exclusion of MUG decreased the number of isolates which pose no risk by 1.3% and increased the number of isolates occurring in the maximum risk for over-estimation category by 1.3%. When comparing the impact of the exclusion of MUG on the amount of no risk isolates for both under- and over-estimation, the net increase in accuracy is 19.3% due to the 20.6% increase in first category isolates for the under-estimation of *E. coli*. When this increase is compared to the 1.3% net increase in maximum risk isolates across under- and over-estimation, it is clear that the exclusion of MUG will still result in a considerable (18.0%) increase in the accuracy of *E. coli* enumeration.

In addition to the improved accuracy, the exclusion of MUG would result in less labour for the analyst and, most importantly, financial gains. At the time of writing, ten vials of MUG supplement cost approximately 800 South African Rands, which would make ten litres of EC broth with MUG which, in turn, would make 1 000 tubes of EC broth with MUG. Another large financial consideration is that the exclusion of MUG negates the need to incur the costs for a UV light to observe fluorescence. These lights can cost upwards from 3 000 South African Rands, depending on their sophistication and additional features. Therefore, based on the results obtained in this study, it is suggested that MUG should be evaluated and considered for exclusion from the MTF assay used to analyse the river waters under investigation as part of the overarching project. However, given the limited numbers, and sites of origin, of isolates used to conduct this risk classification, a conclusive recommendation to remove MUG from MTF cannot be made here. This recommendation arises predominantly from the proven increase in accuracy, but carries the additional benefit of cost reduction.

### The influence of L-EMB agar on the accuracy of the MTF method

The exclusion of L-EMB agar from the MTF assay also increased the number of isolates which did not pose any risk for under-estimation of *E. coli*. Although the increase was, admittedly, lower than that induced by the exclusion of MUG, it still resulted in a decrease in the possibility of any degree of under-estimation to 44.1%. Furthermore, the exclusion of L-EMB agar also reduced the proportion of isolates in high risk categories by 2.9%. When L-EMB agar was excluded from the assay, the number of isolates which posed no risk and the maximum risk for over-estimation remained unchanged. However, the proportion of isolates in low risk categories did decrease by 7.1%. Although this change deserves consideration, all categories which do not describe zero risk for over-estimation should be considered to carry potential risk and should, therefore, carry less weight than categories which pose zero risk. For this reason, the reshuffling of low and high risk isolates within the over-estimation risk classification was considered to be of little consequence when compared to the benefit of increasing the net number of isolates which pose no risk for under- or over-estimation by 5.9% with no corresponding increase in maximum risk isolates for either risk.

Since the exclusion of L-EMB agar from the method resulted in a very small net increase in accuracy for the enumeration of *E. coli*, the cautious recommendation to exclude the medium from the MTF method is far less compelling when compared to the recommendation made regarding the exclusion of MUG. The recommendation in the latter case was predominantly based on the scientific consideration of accuracy. In the case of L-EMB agar, however, the accuracy will be increased much less by the omission of the medium. However, since the inclusion or exclusion made very little difference to the accuracy of the method, financial considerations become of greater importance. The price of 500 g of this agar is currently approximately 630 South African Rands, and this implication along with reduced labour and a 5.9% net increase in no risk isolates is sufficient evidence to support this recommendation. In addition, and very importantly, the exclusion of the confirmatory step on L-EMB agar will reduce the time until *E. coli* enumeration results are available from six to five days in cases where MTF analysis is necessitated or preferred above more rapid methods such as *E. coli* specific quantitative PCR.

## 8.6 REFERENCES

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## CHAPTER 9

### GENERAL DISCUSSION AND CONCLUSION

#### 9.1. INTRODUCTION

It is of fundamental importance that water used for the irrigation of produce intended for human consumption is microbiologically safe. This is especially true for the production of minimally processed foods (MPFs) which are likely to be consumed raw after receiving minimal processing, like fruits and salad vegetables. This category of food products poses an elevated risk for the health of the consumer when irrigated with faecally contaminated water. Pathogens present in the water may be transferred onto plant surfaces or internalised via the soil or wounds in the plant tissue, with little or no subsequent removal through processing. For these reasons, it is important that farmers and food producers are aware of the microbiological state of irrigation water used to grow MPFs, to ensure that timely remedial interventions are implemented when water is shown to be faecally contaminated. Despite the existence of a wide variety of indicators which can be used to indicate faecal contamination in water, *Escherichia coli* is considered to be the best (Edberg *et al.*, 2000). Coliforms, previously used as faecal indicator, are also sporadically used as indicators of the efficacy of water chlorination (Ashbolt *et al.*, 2001), a step which is often used to disinfect water before irrigation of MPFs. Several methods for detecting and enumerating coliforms and *E. coli* have been developed, each with their own benefits and drawbacks. During this research, an important South African source of irrigation water, the Berg River, was monitored. The main objective, however, was to critically evaluate the accuracy of methods for enumerating coliforms and *E. coli*.

#### 9.2. GENERAL DISCUSSION AND CONCLUSION

##### 9.2.1. The Berg River – baseline quality and appropriateness for irrigation of MPFs

###### Microbiological quality

The baseline determination of the quality of the Berg River showed that the river consistently carried levels of bacteria above those that could be expected from environmental sources such as animals (USGS, 2007) alone. These bacteria included the faecal indicator *E. coli*, the levels of which were found to be outside the World Health Organization (WHO) guideline for the irrigation of crops likely to be eaten raw (WHO, 1989) in 20.5% of samples. Analyses to determine the absence or presence of *Salmonella* spp. also indicated large proportion of samples from all sites in the Berg River carried *Salmonella*. The combined high levels of the faecal indicator with a high frequency of *Salmonella* presence is troubling for the microbiological quality of fresh produce which

is irrigated with this water. While the practice of chlorinating water before irrigation does decrease the number of bacteria present in the water, the reduction may not be sufficient to render highly polluted water safe. Chlorination may also result in increasingly severe problems over the long term, with the emergence of antibiotic-resistant bacteria (Murray *et al.*, 1984) as well as disinfectant by-products. Nevertheless, the irrigation of fresh produce is an essential part of producing these crops in South Africa and abstaining from irrigation altogether is not a realistic recommendation. Therefore, chlorination is currently the only treatment being implemented (albeit on a limited scale) to deal with the microbiological contamination of water from the Berg River while more sustainable long-term strategies are investigated. Such strategies should include the installation of sanitation infrastructure in unserved areas, as well as improvements and maintenance of ageing infrastructure and wastewater treatment works. These steps will reduce that amount of faecal pollution reaching environmental water sources and result in an increase in irrigation water quality. However, these interventions will require the co-operation of municipalities and the Department of Water Affairs (DWA). The DWA should, by the powers vested in them by the National Water Act of 1998 (South African Government, 1998), further monitor the progress of interventions and the state of water resources to determine whether improvements can be seen in water quality.

#### Physico-chemical quality

In terms of physico-chemical parameters, the detection of low pH and elevated chemical oxygen demand (COD) levels in the Berg River were considered problematic. However, irrigation with water of a low pH poses no health risk to the consumer as is the case with pathogens present, but can result in a decrease in the lifetime of the irrigation network through corrosion (Bauder *et al.*, 2003). Essentially, elevated COD levels pose no health risk either, but an increase in COD does indicate pollution in the river. There is an urgent need for responsible and accountable practices by industries discharging industrial effluent into rivers. It is recommended that these discharges be regularly tested to ensure that the effluent is of an acceptable standard according to the various guidelines, and should be regularly and actively scrutinised by DWA. If the effluent is not of an acceptable quality, timely measures should be implemented to treat the effluent before discharge to minimise damage and health risks.

#### **9.2.2. Evaluation of traditional and rapid alternative methods for the enumeration of coliforms and *E. coli***

##### Importance of determination of faecal contamination

Since the practice of irrigating from rivers cannot be prevented for practical and financial reasons, it is recommended that the levels of faecal bacteria present in river water are continuously



monitored. This information will be crucial to food producers, who should be informed of instances when produce was irrigated with polluted water. This will afford the food industry the opportunity to take responsible remedial measures, e.g. the disinfection of produce with chlorinated water, or using unacceptably contaminated produce in processed products. For this monitoring purpose, the MTF method is one method recognised and recommended by regulatory bodies due to its statistical semi-quantification of coliform and *E. coli* numbers. However, this method is time-consuming and may not produce results in a manner which is timely enough to prevent the irrigation of MPFs with polluted water. In terms of the critical evaluation of enumeration methodology for coliforms and *E. coli*, four methodological approaches were evaluated: an in-depth evaluation of factors potentially interfering with the accuracy of MTF through the study of isolates which result in correct enumeration and those resulting in false positive and false negative enumerations; the use of microbiological or physicochemical parameters to estimate *E. coli* numbers which had been determined by MTF; a comparison of Colilert-18 as a rapid alternative for MTF, and finally; an evaluation of polymerase chain reaction (PCR) protocols to distinguish *E. coli* from coliforms and differentiate between five diarrheagenic pathotypes of *E. coli*.

#### Critical evaluation of MTF as recommended enumeration method

The MTF method is considered to be one of the most accurate methods in the enumeration of coliforms and *E. coli*. This is mainly attributable to the large number of selective hurdles in the method, as well as a statistical approach of semi-quantification. Therefore, despite the fact that this method cannot produce results in less than two to four days, it is used in industry and for legal proceedings when high accuracy is required. The perceived high level of accuracy was evaluated during this research, and was found to be justified. However, it was also determined that the single largest factor leading to inaccuracies in the enumeration of coliforms from river water was the presence of false negative coliforms which could not produce gas from lactose. Inaccuracies in the enumeration of *E. coli* were caused both by false negative and false positive reactions. The factors contributing to the occurrence of such reactions were failure by *E. coli* strains to produce gas from lactose or hydrolyse 4-methylumbelliferyl- $\beta$ -D-glucuronidase (MUG), and the ability of non-*E. coli* isolates to hydrolyse MUG. These problematic factors were the first indications which lead to the question of whether MUG was beneficial or detrimental to the accuracy of MTF. No work was done during this study to determine the proportion of problematic isolates present in the sample, and it is recommended that further work be done in order to ascertain the magnitude of the problems caused by these organisms.

One of the greatest problems with MTF, which was identified during various stages of this research, was the inaccuracies brought about by the “grab sampling” technique. These inaccuracies were made apparent when it was discovered that some isolates could not reproduce the MTF reaction from which they were initially isolated. This was also confirmed by the molecular work, which showed that some isolates did not possess the genes necessary for the phenotypic

reactions in MTF from which they were obtained. This is troublesome for the accuracy of the method, since the transfer of organisms from one step to the next takes the form of a grab sample. This problem is intrinsic to the method and cannot be removed, but it is recommended that further research is done to determine the extent of the inaccuracy caused by this.

Dendrograms may provide information regarding the similarity or dissimilarity of organisms which remains hidden without mathematical analysis. The numerical data which was obtained during the characterisation and identification of MTF isolates was used to construct similarity and dissimilarity dendrograms to ascertain whether isolates with similar or identical MTF reactions would be grouped together based on their characteristics or ribosomal protein spectra. The results were inconclusive, with no grouping of isolates with similar MTF reactions. This is attributable to the complexity of phenotypic reactions displayed by organisms during MTF. These reactions are also dependent on variables such as environmental adaptation, making them far too complex to be predicted by API 20E reactions or ribosomal spectra. One interesting result was the observation that isolates which could not ferment lactose during MTF could ferment a greater variety of other carbohydrates, and it was hypothesised that these isolates used alternative carbohydrates preferentially over lactose. It is recommended that this be investigated experimentally in greater detail. If this is found to be the case, alternative carbohydrates may hold potential for refining the MTF analysis.

Due to the highly unpredictable outcomes observed in isolates which were obtained from MTF through the “grab sample” technique, isolates were analysed individually with MTF to determine their true reactions. These reactions were used in a novel qualitative methodological risk classification system to determine the number of isolates which would result in problematic reactions with MTF, as well as the severity of these problematic reactions. The results indicated that even with a high proportion of problematic isolates in the group, 50.5% of isolates posed no risk for the under-estimation of coliforms. Isolates causing the over-estimation of coliforms were rare, and are not considered to impact greatly on the method due to several inhibitory substances present in MTF media. For the enumeration of *E. coli*, 50.0% of isolates posed no risk for the under-estimation of *E. coli* while only 45.5% of non-*E. coli* isolates posed no risk for over-estimation. These results are somewhat disquieting and raise concerns regarding the accuracy of *E. coli* enumeration with MTF. However, it is recommended that in future research the proportion of problematic *E. coli* and non-*E. coli* organisms present during a full MTF analysis is determined through large-scale laboratory analyses. This should not involve the selection of MTF reaction tubes for the isolation of organisms, but should include all MTF reaction tubes and all organisms present therein. These isolates should be subjected to a similar individual MTF analysis to determine their true MTF reactions before assigning a “problematic” status. Only then the true proportion of these problematic organisms will be known, and can be used to transform qualitative information with the methodological risk assessment into quantitative information, i.e. how many organisms will cause an incorrect reaction of a certain severity.

MUG and Levine-eosin methylene blue (L-EMB) agar is used during MTF analyses to specifically test and confirm, respectively, the presence of *E. coli*. To determine their influence on the accuracy of the method, the qualitative methodological risk classification was applied to the isolate set using criteria which excluded MUG and L-EMB. The results showed that the exclusion of MUG from the analysis would lead to an increased (20.6%) detection of *E. coli* isolates (those which cannot hydrolyse MUG), but a slight increase (1.3%) in the detection of non-*E. coli* isolates which could grow at 44.5°C but are unable to fluoresce with MUG. These surprising results show that MUG may not always increase the accuracy of the MTF analysis. This may not always be the case due to differences in bacterial composition in the sample and it is recommended that, for routine samples, the general bacterial composition of the sample type be determined as a verification step before deciding to include or exclude MUG from the analysis. However, this would only be necessary in sample types where a large proportion of the bacterial population are suspected of being MTF-atypical, a scenario which is not considered to be common in nature. The results for L-EMB agar were less conclusive and resulted in small increases in accuracy through a reduction of *E. coli* under-estimation, which were offset by similar increases in the risk of over-estimation. Therefore, the recommendation based on this work would be to retain L-EMB agar as a validation step for *E. coli*.

#### Evaluation of microbiological and physico-chemical measurements as alternative method

The use of microbiological and physico-chemical measurements, which are faster and easier to obtain, to estimate *E. coli* numbers in the same sample was evaluated with results from the Berg River. Not individual measurements, nor the combined set of parameters (multiple  $R^2=0.496$ ), could estimate MTF-obtained *E. coli* numbers accurately. In addition, one of the measurements which was used during this evaluation was COD, which is often used by wastewater treatment works to determine whether effluent is of an acceptable microbiological quality. This parameter showed inverse and weak ( $r^2 = 0.01$ ) correlation with *E. coli* numbers. Therefore, it is strongly recommended that the practice of using COD values as process indicators for the removal of faecal pollution be discontinued. While these measurements would give results in less time than direct *E. coli* enumeration, the results are highly inaccurate and this approach would be strongly discouraged to estimate *E. coli* numbers in a sample.

#### Evaluation of Colilert-18 as alternative method

The comparison of Colilert-18 enumeration values with coliform and *E. coli* numbers determined with MTF indicated that this method shows promise as a rapid alternative to MTF. While Colilert-18 tended towards higher enumerations of coliforms and lower enumerations of *E. coli* the method showed acceptable and fair correlations, respectively, with MTF. However, this agreement between the two methods was shown to become weaker when samples were highly polluted.

When coliform levels surpassed 100 000 coliforms MPN.100 mL<sup>-1</sup> and *E. coli* numbers were higher than 50 000 *E. coli* MPN.100 mL<sup>-1</sup>, Colilert-18 became increasingly prone towards higher coliform and lower *E. coli* enumerations. However, when such high levels of faecal pollution are encountered in a sample, the slight under- or over-estimation of coliform and *E. coli* numbers will not impact on results so adversely that the exceeding of safe irrigation guidelines for MPFs remains undetected. In addition, food producers can be alerted of irrigation with polluted water much faster, resulting in more rapid corrective action, since Colilert-18 produces results after 18 hours. This time gain will offset the relatively small compromise on enumeration accuracy. In the light of these considerable time gains it is recommended that Colilert-18 be used for the monitoring of irrigation water, since the irrigation of MPFs cannot be avoided.

#### Evaluation of *E. coli*-specific PCR as alternative method

PCR is considered to be a rapid method for the detection and enumeration of organisms, since the method does not require culturing before analysis. PCR protocols have been developed for the detection of coliforms and *E. coli* through the detection of a variety of group- or organism-specific genes. The ability of these protocols to detect coliform and *E. coli* environmental isolates obtained from river water were evaluated during this research, and it was found that the protocol for detecting coliforms through the detection of the *lacZ* gene could not detect a variety of coliform isolates with primer annealing at 59.7°C. Literature suggests that annealing at 40.0°C may improve results (Bej *et al.*, 1990), and it is recommended that protocol development with annealing at this temperature is explored to determine whether the adjusted protocol could be used to detect coliform isolates. However, the implication of these results was that the focus was shifted to distinguish between *E. coli* and non-*E. coli* isolates. Detecting *E. coli* through the use of *uidA* and *tuf* genes showed that both protocols were effective in distinguishing *E. coli* from non-*E. coli*. However, detection with *uidA* showed higher accuracy than detection with *tuf*, which incorrectly detected two isolates of *Enterobacter* as *E. coli*. Since detection of the *uidA* gene in *E. coli* shows promise for the specific and ubiquitous detection of *E. coli*, it is recommended that further research is done to develop a quantitative PCR (qPCR) protocol for enumerating *E. coli* directly from water. Such a protocol could potentially reduce the time until results are available to ca. two hours (the current *uidA* protocol for PCR and electrophoresis takes 1.5 hours), which would lead to even faster reaction times to counteract the health risks posed by MPFs which has been irrigated with faecally contaminated water. However, these theoretical gains in time do not take into account the high probability that such a protocol would require either pre-enrichment or sample concentration before enumeration can be performed.

The differentiation of diarrheagenic pathotypes of *E. coli* from commensal strains is important when a more comprehensive analysis of the risk posed by irrigation water is required. The multiplex PCR protocol evaluated during this study was successful in the detection of all five pathotypes tested for and could be useful when more information is required regarding the nature

of a faecally polluted sample. However, while the protocol is rapid in comparison to traditional culturing methods, it is relatively time-consuming and costly in relation to other PCR protocols. In addition, the protocol as it currently exists cannot enumerate these pathotypes. For these reasons, the method is not recommended for the routine assessment of irrigation water.

### **9.2.3. Comparison of API and MALDI-TOF MS for the identification of environmental isolates**

Due to the environmental origin of the isolates used during this study, which presumably resulted in adapted strains with biochemical reactions divergent from their clinical counterparts, biochemical identification with API 20E was unsuccessful for 18 (16.2%) of the isolates. In addition, 18.9% of the isolates were misidentified based on biochemical reactions and were reassigned to different genera by MALDI-TOF MS. These findings highlighted some limitations of the API 20E system, particularly in the identification of environmental isolates, while simultaneously emphasising the ability of the MALDI-TOF MS-based method to identify isolates despite any environmental adaptations. Based on these results alone, the MALDI-TOF MS method would be strongly recommended for isolate identification. However, the capital investment and running costs of this system does not at present make it a feasible alternative to the relatively cheap API system, which produces acceptably accurate results for a fraction of the cost.

## **9.3. CONCLUDING REMARKS**

The work done in this study which focused on the state of the upper Berg River found evidence of both faecal and chemical contamination in this river. From the food science perspective, these findings are cause for concern as this is an important source of irrigation water for the region. While these results motivate the strong recommendation that various role-players become actively involved in correcting this situation for the benefit of the economy, the ecology, the health of individuals coming into contact with the river, as well as the food industry; it is conceded that such interventions will not materialise overnight. This reality highlights the importance of interim strategies to minimise the detrimental impact of irrigation with polluted water, one of which is monitoring of the levels of faecal contamination in river water. In addition, it is important that the link between microbiologically polluted irrigation water and contaminated fresh produce be further established to motivate various role-players to induce changes to the situation.

The second phase of this study was focused on critically evaluating methodologies for detecting and enumerating coliforms and *E. coli*, as decision-making to minimise the impact of irrigation with polluted water can only be as good as the information it is based on. After a multi-faceted and in-depth evaluation of the MTF method it was found to be acceptably accurate at detecting target organisms and excluding non-target organisms, however, several problematic areas were identified. While some of these limitations are intrinsically part of the method (such as

the “grab sample” transfer technique), others may be alleviated through further research. Furthermore, evaluation of the MTF method led to the important discovery that exclusion of the MUG reagent can reduce inaccuracy associated with the enumeration of *E. coli*.

The evaluation of rapid alternatives to enumerated coliforms and *E. coli* indicated that microbiological and physico-chemical measurements used to assess the quality of water could not accurately predict the numbers of *E. coli* present in the water, and should not be used for this purpose. However, Colilert-18 was found in this work to be an acceptable alternative to MTF for the monitoring of faecal pollution in rivers due to the acceptable and fair correlations which were empirically demonstrated for coliform and *E. coli* enumerations, respectively. In addition, it was also determined that variations in pollution levels did not adversely affect these correlations, except at very high coliform and *E. coli* concentrations. These findings, in combination with a reduction in time before results are available, are important as they scientifically confirm that Colilert-18 can be used for the rapid determination of faecal contamination levels in river water. It is recommended that other established and emerging rapid methods of quantification should also be evaluated in future studies. For example, the single-vial resazurin tests, which indicates microbial growth in a small volume of sample after approximately 24 (Sarker *et al.*, 2007) to 72 (Reinheimer & Demkow, 1990) hours, despite being marginally less rapid than Colilert-18, may be more practical for produce growers since detection does not require a spectrophotometer (Sarker *et al.*, 2007) or UV light source (as is the case with Colilert-18). *E. coli*-specific PCR protocols were also evaluated and detection based on *uidA* was found to have high accuracy in the detection of environmental strains of *E. coli*. These results are compelling, and should give the necessary impetus for the development of a method which combines the speed and accuracy of *E. coli*-specific PCR detection with quantification. If this is accomplished, faecal contamination levels may be determined at even greater speed, which will lead to more timely corrective action to minimise the health risks associated with irrigation of MPFs with contaminated water.

#### 9.4. REFERENCES

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